I

Biochemical and Biophysical Research Communications 285, 1217–1219 (2001)
doi:10.1006/bbrc.2001.5308, available online at http://www.idealibrary.com on IDEAL®

Preconditioning Decreases Ischemia/Reperfusion-Induced Peroxynitrite Formation

Csaba Csonka,* Tamás Csont,† Annamária Ónody,* and Péter Ferdinandy*,[1]

*Cardiovascular Research Group, Department of Biochemistry, University of Szeged, Szeged, Hungary; and †Department of Pharmacology, Cardiovascular Research Group, University of Alberta, Edmonton, Alberta, Canada

Received June 25, 2001

The role for peroxynitrite ($ONOO^-$) in the mechanism of preconditioning is not known. Therefore, we studied effects of preconditioning and subsequent ischemia/reperfusion on myocardial $ONOO^-$ formation in isolated rat hearts. Hearts were subjected to a preconditioning protocol (three intermittent periods of global ischemia/reperfusion of 5 min duration each) followed by a test ischemia/reperfusion (30 min global ischemia and 15 min reperfusion). When compared to nonpreconditioned controls, preceding preconditioning improved postischemic cardiac performance and significantly decreased test ischemia/reperfusion-induced formation of free nitrotyrosine measured in the perfusate as a marker for cardiac endogenous $ONOO^-$ formation. During preconditioning, however, the first period of ischemia/reperfusion increased nitrotyrosine formation, which was attenuated after the third period of ischemia/reperfusion. We conclude that classic preconditioning inhibits ischemia/reperfusion-induced cardiac formation of $ONOO^-$ and that subsequent periods of ischemia/reperfusion result in a gradual attenuation of ischemia/reperfusion-induced $ONOO^-$ generation. This mechanism might be involved in ischemic adaptation of the heart.

Key Words: **peroxynitrite; nitrotyrosine; preconditioning; ischemia/reperfusion; rat heart.**

Although nitric oxide (NO) has been shown to contribute to the development of cardiac stress adaptation, the exact role of NO in myocardial ischemia/reperfusion and ischemic preconditioning is not completely known (see 1–3 for reviews). Myocardial ischemia leads to an accumulation of NO which might contribute to ischemia/reperfusion injury (4–7). We have previously shown that classic preconditioning decreases the harmful accumulation of NO during ischemia/reperfusion in rat hearts (5). Recent studies suggest that the harmful effects of NO in the heart and in the vasculature are due to the formation of peroxynitrite ($ONOO^-$), a toxic reaction product of NO and superoxide (7–9), rather than the possible toxic effect of NO itself. It is known that cardiac production of superoxide radical is accelerated upon reperfusion, therefore, simultaneous production of NO and superoxide may favour the formation of $ONOO^-$ during ischemia/reperfusion (7–9).

The possible role for $ONOO^-$ in preconditioning is not known. Altug *et al.* have found that exposure of isolated rat hearts to 1 μM final concentration of exogenous $ONOO^-$ was capable of mimicking the beneficial effects of ischemic preconditioning in rat hearts (10, 11). As exogenous $ONOO^-$ does not reflect the effect of endogenous $ONOO^-$ formation (14), here we measured endogenous $ONOO^-$ formation during preconditioning and subsequent ischemia/reperfusion in isolated working rat hearts to clarify the possible contribution of endogenous $ONOO^-$ to ischemic preconditioning.

METHODS

The investigation conforms the Guide for the care and use of laboratory animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1985) and was approved by local ethics committees.

Isolated rat heart preparation. Male Wistar rats (300–350 g) were anesthetized with diethylether and given 500 U/kg heparin. Hearts were then isolated and perfused in Langendorff or working mode with an oxygenated, normothermic Krebs-Henseleit buffer supplemented with 0.3 mM L-tyrosine (13). Myocardial functional parameters and lactate dehydrogenase (LDH) release were measured as described (5).

Experimental protocol. A nonpreconditioning and a preconditioning protocol were applied before induction of test ischemia/reperfusion as described in detail (5). After 10 min equilibration, preconditioning was induced by three intermittent cycles of 4.75 min no-flow ischemia, separated by 0.5 min Langendorff perfusion followed by 4.75 min aerobic working perfusion. The 0.5 min Langendorff perfusion allowed for the spontaneous restoration of sinus rhythm before switching to working mode between no-flow periods.

[1] To whom correspondence should be addressed at Cardiovascular Research Group, Department of Biochemistry, University of Szeged, Faculty of Medicine, Dóm tér 9, Szeged, Hungary, H-6720. Fax: (+36) 62 545 097. E-mail: Peter@bioch.szote.u-szeged.hu.

0006-291X/01 $35.00

TABLE 1

Aortic Flow, Left Ventricular End-Diastolic Pressure, and Lactate Dehydrogenase Release in Nonpreconditioned and Preconditioned Hearts before and after Test Ischemia and Reperfusion

	AF (mL/min)	LVEDP (kPa)	LDH (mU/min/g)
Before test ischemia/reperfusion			
Nonpreconditioned	45.9 ± 1.2	0.54 ± 0.05	n.d.
Preconditioned	43.6 ± 2.1	0.52 ± 0.05	n.d.
After ischemia/reperfusion			
Nonpreconditioned	16.4 ± 1.1	1.76 ± 0.05	163.0 ± 11.6
Preconditioned	25.1 ± 1.6*	1.34 ± 0.08*	63.9 ± 12.3*

Note. n.d., nondetectable; *$P < 0.05$ vs corresponding nonpreconditioned group. $n = 7$ in each group.

Nonpreconditioned control and preconditioned hearts were then subjected to 30 min global no-flow ischemia followed by 15 min reperfusion.

Measurement of nitrotyrosine, a marker for ONOO⁻ in the perfusate. $ONOO^-$ promotes nitration of phenolic compounds such as tyrosine, the nitration of which leads to the formation of a stable product, 3-nitrotyrosine (12). Therefore, to measure cardiac $ONOO^-$ generation, Krebs-Henseleit buffer was supplemented with 0.3 mM L-tyrosine and free nitrotyrosine formation was detected in the perfusate as a marker of myocardial $ONOO^-$ generation (7, 13). Tyrosine at 0.3 mM does not affect cardiac mechanical function (7). Free nitrotyrosine concentration was measured before preconditioning, after the first and third no-flow period of preconditioning, as well as following test ischemia. Samples of coronary effluent during Langendorff perfusion periods were collected for 30 s and stored in −80°C until assayed for nitrotyrosine. Free nitrotyrosine was measured by ELISA (Cayman Chemicals, Ann Arbor, MI) as described (13). Briefly, perfusate samples were incubated overnight with anti-nitrotyrosine rabbit IgG and nitrotyrosine acetylcholinesterase tracer in precoated (mouse anti-rabbit IgG) microplates followed by development with Ellman's reagent. Nitrotyrosine formation was normalized to coronary flow and wet weight of the hearts and expressed as pmol/min/g.

Statistics. Data were expressed as means ± SEM and analyzed with ANOVA. $P < 0.05$ was accepted as a statistically significant difference.

RESULTS

In nonpreconditioned control hearts, test ischemia/reperfusion resulted in a marked decrease in aortic flow (AF) and a considerable increase in left ventricular end-diastolic pressure (LVEDP) and LDH release (Table 1). When preconditioning was applied before test ischemia, postischemic AF increased and LVEDP and LDH release decreased showing the protective effect of classic preconditioning against acute ischemia/reperfusion injury (Table 1).

In the nonpreconditioned control group, test ischemia/reperfusion markedly increased cardiac nitrotyrosine formation. Preceding preconditioning with three brief periods of no-flow ischemia significantly decreased test ischemia/reperfusion-induced nitrotyrosine formation (Fig. 1). In the preconditioned group, however, the first cycle of 4.75 min no-flow ischemia followed by reperfusion markedly increased nitroty-

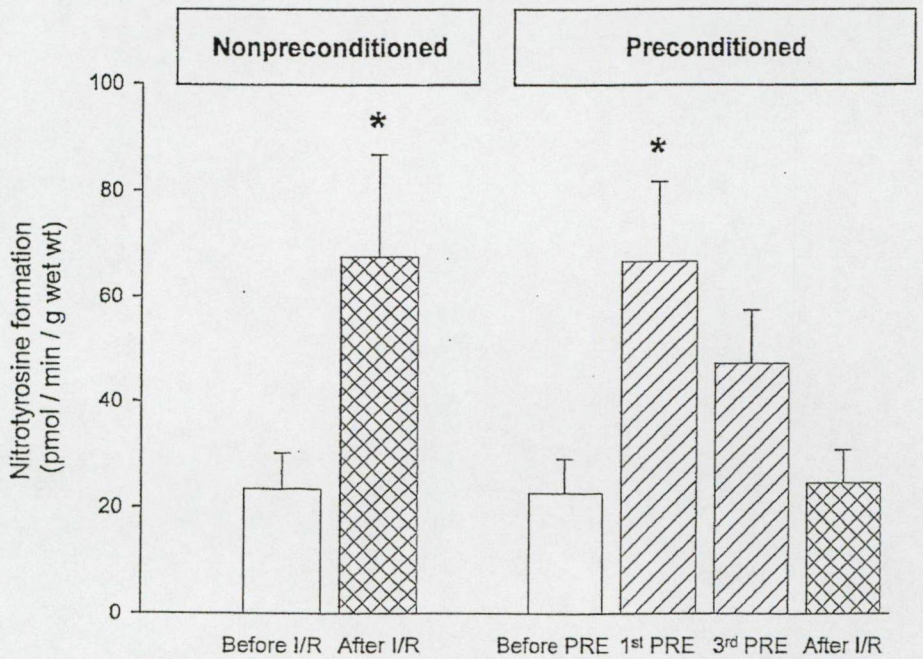


FIG. 1. Effect of preconditioning and test ischemia/reperfusion on cardiac formation of free nitrotyrosine, a marker for endogenous peroxynitrite formation. Nitrotyrosine was measured in the perfusate by ELISA. PRE, preconditioning; I/R, ischemia/reperfusion. *$P < 0.05$ vs corresponding "before I/R" or "before PRE" values.

rosine formation, which was attenuated after the third cycle of no-flow ischemia (Fig. 1).

DISCUSSION

This is the first demonstration that preceding ischemic preconditioning inhibits endogenous cardiac $ONOO^-$ production induced by test ischemia/reperfusion, and that subsequent cycles of ischemia/reperfusion result in a gradual decrease in $ONOO^-$ formation.

It is well established that myocardial ischemia leads to an accumulation of NO in the heart which is associated with a deterioration of myocardial mechanical function during reperfusion. We have previously found that ischemic preconditioning markedly decreases NO accumulation during test ischemia and reperfusion, improves postischemic cardiac function, and decreases the release of LDH (5). As many studies suggest that NO-dependent myocardial injury is mediated by $ONOO^-$ (4, 6, 7), and that $ONOO^-$ formation deteriorates myocardial mechanical function (13), we hypothesized that ischemic preconditioning attenuates $ONOO^-$ formation, thereby protecting the heart against functional damage. Accordingly, we have found here that ischemic preconditioning markedly decreased $ONOO^-$ formation upon ischemia/reperfusion.

Although preconditioning attenuated $ONOO^-$ formation upon test ischemia/reperfusion in our present study, the first brief cycle of preconditioning ischemia/reperfusion significantly enhanced $ONOO^-$ formation, however, after the third cycle of ischemia/reperfusion $ONOO^-$ formation was reduced. This may show that $ONOO^-$ formed during ischemia/reperfusion might act as a trigger for preconditioning, but preconditioning in turn decreases formation of $ONOO^-$ upon subsequent cycles of ischemia/reperfusion. $ONOO^-$ as a trigger for preconditioning is supported Altug *et al.* (10), who described that infusion of exogenous $ONOO^-$ at 1 μM final concentration into the perfusion buffer is able to mimic the effects of ischemic preconditioning in isolated crystalloid perfused rat hearts, which was abolished by the administration of the antioxidant *N*-2-mercaptopropionylglycine.

In conclusion, our present study is the first demonstration that ischemic preconditioning decreases ischemia/reperfusion-induced endogenous formation of $ONOO^-$. This mechanism might be involved in preconditioning-induced cardioprotection.

ACKNOWLEDGMENTS

This work was supported by grants from Hungarian Scientific Research Fund (T029843), Hungarian Ministries of Education (FKFP-0340/2000 and FKFP 0057/2001), and Health (ETT 51/2000).

REFERENCES

1. Bolli, R. (2000) The late phase of preconditioning. *Circ. Res.* **87**, 972–983.
2. Ferdinandy, P., Szilvassy, Z., and Baxter, G. F. (1998) Adaptation to myocardial stress in disease states: Is preconditioning a healthy heart phenomenon? *Trends. Pharmacol. Sci.* **19**, 223–229.
3. Przyklenk, K., and Kloner, R. A. (1998) Ischemic preconditioning: Exploring the paradox. *Prog. Cardiovasc. Dis.* **40**, 517–547.
4. Zweier, J. L., Wang, P., and Kuppusamy, P. (1995) Direct measurement of nitric oxide generation in the ischemic heart using electron paramagnetic resonance spectroscopy. *J. Biol. Chem.* **270**, 304–307.
5. Csonka, C., Szilvassy, Z., Fulop, F., Pali, T., Blasig, I. E., Tosaki, A., Schulz, R., and Ferdinandy, P. (1999) Classic preconditioning decreases the harmful accumulation of nitric oxide during ischemia and reperfusion in rat hearts. *Circulation* **100**, 2260–2266.
6. Schulz, R., and Wambolt, R. (1995) Inhibition of nitric oxide synthesis protects the isolated working rabbit heart from ischaemia-reperfusion injury. *Cardiovasc. Res.* **30**, 432–439.
7. Yasmin, W., Strynadka, K. D., and Schulz, R. (1997) Generation of peroxynitrite contributes to ischemia-reperfusion injury in isolated rat hearts. *Cardiovasc. Res.* **33**, 422–432.
8. Beckman, J. S., and Koppenol, W. H. (1996) Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and ugly. *Am. J. Physiol.* **271**, C1424–C1437.
9. Wang, P., and Zweier, J. L. (1996) Measurement of nitric oxide and peroxynitrite generation in the postischemic heart. Evidence for peroxynitrite-mediated reperfusion injury. *J. Biol. Chem.* **271**, 29223–29230.
10. Altug, S., Demiryurek, A. T., Kane, K. A., and Kanzik, I. (2000) Evidence for the involvement of peroxynitrite in ischaemic preconditioning in rat isolated hearts. *Br. J. Pharmacol.* **130**, 125–131.
11. Altug, S., Demiryurek, A. T., Ak, D., Tungel, M., and Kanzik, I. (2001) Contribution of peroxynitrite to the beneficial effects of preconditioning on ischaemia-induced arrhythmias in rat isolated hearts. *Eur. J. Pharmacol.* **415**, 239–246.
12. van der Vliet, A., Eiserich, J. P., O'Neill, C. A., Halliwell, B., and Cross, C. E. (1995) Tyrosine modification by reactive nitrogen species: A closer look. *Arch. Biochem. Biophys.* **319**, 341–349.
13. Ferdinandy, P., Daniel, H., Ambrus, I., Rothery, R. A., and Schulz, R. (2000) Peroxynitrite is a major contributor to cytokine-induced myocardial contractile failure in rat hearts. *Circ. Res.* **87**, 241–247.
14. Ferdinandy, P., and Schulz, R. (2001) Peroxynitrite: Toxic or protective in the heart? *Circ. Res.* **88**, E12–E13.

II

Effect of classic preconditioning on the gene expression pattern of rat hearts: a DNA microarray study

Annamária Ónody[a], Ágnes Zvara[b], László Hackler Jr.[b], László Vígh[c], Péter Ferdinandy[a], László G. Puskás[b,*]

[a] *Cardiovascular Research Group, Department of Biochemistry, University of Szeged, Dóm tér 9, H-6720 Szeged, Hungary*
[b] *Laboratory of Functional Genomics, Biological Research Center, Hungarian Academy of Sciences, P.O. Box 521, H-6701 Szeged, Hungary*
[c] *Department of Biochemistry, Biological Research Center, Hungarian Academy of Sciences, Temesvári krt. 62, H-6726 Szeged, Hungary*

Received 23 September 2002; revised 13 December 2002; accepted 3 January 2003

First published online 14 January 2003

Edited by Veli-Pekka Lehto

Abstract To profile gene expression patterns involved in ischemic preconditioning, we monitored global gene expression changes by DNA microarray analysis of 3200 rat-specific genes and by real-time quantitative polymerase chain reaction in rat hearts. Forty-nine genes with altered expression were found after ischemia/reperfusion as compared to control non-ischemic hearts and 31 genes were characteristic for classic preconditioning followed by ischemia/reperfusion as compared to ischemia/reperfusion without preconditioning. Genes with altered expression due to ischemia and/or preconditioning included those controlling protein degradation, stress responses, apoptosis, metabolic enzymes, regulatory proteins, and several unknown cellular functions. Metallothionein, natriuretic peptides, coagulation factor VII, cysteine proteinase inhibitor, peroxisome proliferator activator receptor γ and myosin light chain kinase genes were previously suspected to be related to several cardiovascular diseases, however, most of these genes have not previously been shown to be related to myocardial ischemia/reperfusion. Some genes were observed to change specifically in response to preconditioning: oligoadenylate synthase, chaperonin subunit ε, a cGMP phosphodiesterase (PDE9A1), a secretory carrier membrane protein, an amino acid transporter, and protease 28 subunit. None of these genes has previously been shown to be involved in the mechanism of preconditioning.

Key words: Oligoadenylate synthase; cGMP phosphodiesterase; Metallothionein; Natriuretic peptide; Coagulation factor VII; Cysteine proteinase inhibitor; Peroxisome proliferator activator receptor γ; Chaperonin subunit ε; Myocardial ischemia; Preconditioning

1. Introduction

Ischemic heart disease is a major cause of mortality in the western world. Effective therapeutic strategies for protecting the ischemic myocardium are much sought after. Ischemic preconditioning is a well-described adaptive response in which brief exposure to ischemia markedly enhances the ability of the heart to withstand a subsequent ischemic injury (see [1] for review). Preconditioning confers a remarkable cardioprotection in a variety of species including humans (see [1,2] for reviews), although the cardioprotective effectiveness of ischemic preconditioning might be attenuated in the heart during aging and some disease states such as hyperlipidemia and diabetes (see [3] for review). Preconditioning can be elicited by different sublethal stress signals, such as brief periods of ischemia, hypoxia, rapid electrical pacing, heat stress, administration of bacterial endotoxin, etc. The cardioprotective effect of preconditioning shows two distinct phases. The early phase (classic preconditioning) is manifested within minutes after the preconditioning stimulus and has a duration of less than 2–3 h. The late phase is characterized by a slower onset (20 h) and a duration of up to 72 h.

The underlying molecular mechanisms of ischemic preconditioning have been extensively investigated in the hope of identifying new rational approaches to therapeutic protection of the ischemic myocardium. In spite of the intensive research in the past nearly 2 decades, the exact biochemical mechanism of preconditioning is still a question of debate due to the complexity of the cellular mechanisms involved in this phenomenon. A variety of substances and ion channels, i.e. adenosine, bradykinin, nitric oxide, superoxide, peroxynitrite, calcitonin gene-related peptide, cGMP, protein kinases, norepinephrine, ATP-sensitive K^+ channels, etc., have been shown so far to play a role both in ischemia/reperfusion injury and in the development of the cardioprotective effect of preconditioning [2,4,5]. However, the traditional biochemical and pharmacological approaches have been insufficient so far to explore the key cellular events in ischemia/reperfusion injury and preconditioning. Recent studies therefore attempted to identify gene activity changes during coronary occlusion in the mouse heart using a mouse cDNA array of 588 genes [6] and in a rat infarction model using cDNA array of about 7000 rat genes [7]. However, still very little is known about the gene expression pattern of the heart in response to ischemia/reperfusion and preconditioning.

Therefore, to profile gene expression patterns associated with ischemia/reperfusion and classic preconditioning, we used cDNA microarrays of 3200 rat genes to monitor transcript levels in rat hearts in the hope of identifying new cellular pathways involved in cardiac ischemia and ischemic adaptation.

2. Materials and methods

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of

*Corresponding author. Fax: (36)-62-432 576.
E-mail address: pusi@nucleus.szbk.u-szeged.hu (L. G. Puskás).

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00006-1

Table 1
Primers used in Q-PCR analysis

Gene product	Forward primer	Reverse primer
β-Actin	TTCAACACCCCAGCCATGT	GCATACAGGGACAACACAGCC
Chaperonin ε	TACAGCTCTGCAGATGAAGGATGCTT	TGACATCCGTAAGCCTGGAGAATCTG
Natriuretic peptide type B	GGACCAAGGCCCTACAAAAGAACTTC	GCCGGAGTCTGCAGCCAGGAGGTC
Anion exchange protein 2	CCATGGGTGGCATCTGTGCCCTC	GCAGTAGGTCCCCAATGACCATGGAG
Metallothionein-II	TCGCCATGGACCCCAACTGCTCCTGTG	GAAGCCTCTTTGCAGATGCAGCCCTG
PPARγ	CGGAAGCCCTTTGGTGACTTTATGG	GATGGGCTTCACGTTCAGCAAGCC
Betaine-homocysteine methyltransferase	GGAACCCAGAGTTGCCACCAGATGG	AAATCCCCTTTCTGGGGCGAGCTCC
Cysteine proteinase inhibitor	GCCACTGCTTGAAAAGAAAACCAATGGG	GGTCAGCTCATCATTTTTGGATTTGTTAG

Health (NIH publication No. 85-23, revised 1996) and was approved by local ethics committees.

2.1. Perfusion protocol of isolated rat hearts

Male Wistar rats (300–350 g) were anesthetized with diethylether and given 500 U/kg heparin. Hearts were then isolated and perfused in Langendorff mode with an oxygenated, normothermic Krebs–Henseleit buffer as described [8]. Three different perfusion protocols were applied ($n = 5$–8 in each group). Hearts were subjected to either a preconditioning or a non-preconditioning protocol followed by test ischemia/reperfusion as described in detail [8,9]. After 10 min equilibration, preconditioning was induced by three intermittent cycles of 5 min no-flow ischemia, separated by 5 min aerobic perfusion. Time-matched non-preconditioned and preconditioned hearts were then subjected to 30 min global no-flow ischemia followed by 120 min reperfusion. A time-matched control group was aerobically perfused for 190 min. Heart rate and coronary flow were monitored throughout the perfusion protocol in all groups [8]. Lactate dehydrogenase release was measured from coronary effluent collected for 5 min at the beginning of the perfusion and at 0–5 min and 115–120 min of reperfusion after 30 min global ischemia and at corresponding periods in controls as described [8]. At the end of the perfusion protocols, hearts from all groups were frozen and powdered with a pestle and mortar in liquid nitrogen.

2.2. RNA preparation

Total RNA was purified from each group (25–25 mg tissue from each heart) with NucleoSpin RNA purification kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. RNA preparations from each group ($n = 3$, randomly selected from each group) were pooled, and their quantities and qualities were assessed by gel electrophoresis and spectrophotometry. Total RNA was used for microarray analysis as well as for reverse transcription quantitative polymerase chain reaction (QRT-PCR).

2.3. Microarrays, probe preparations and hybridizations

Construction and use of microarrays were as described [10]. Briefly, 3200 amplified cDNA inserts from rat heart, kidney, liver and brain libraries were purified with MultiScreen-PCR plate (Millipore), resuspended in 50% dimethyl sulfoxide/water, and arrayed on amino-silanized slides (Sigma-Aldrich, St. Louis, MO, USA) using a MicroGrid Total Array System (BioRobotics, Cambridge, UK) spotter with 16 pins with a 4×4 format. All clones were spotted in duplicate. After printing, DNA was UV crosslinked to the slides (Stratagene, Stratalinker, 700 mJ) and stored at room temperature. Prior to hybridization, the slides were blocked in 1×saline sodium citrate (SSC), 0.2% sodium dodecyl sulfate (SDS), 1% bovine serum albumin for 30 min at 42°C, washed with water and dried with high pressure air. 15 μg total RNA from each sample was amplified by a linear antisense RNA amplification method, and labeled with Cy3 or Cy5 fluorescent dye during reverse transcription as described previously [11]. Briefly, 2 μg of amplified RNA was labeled with 0.4 μM random nonamers, 0.1 mM d(G/T/A)TPs, 0.05 mM dCTP (Amersham Pharmacia Biotech, UK), 20 U RNasin (Fermentas, Vilnius, Lithuania), 1×first strand buffer, 200 U RNase H (−) point mutant M-MLV reverse transcriptase (Fermentas), and 0.05 mM Cy3-dCTP or Cy5-dCTP (NEN Life Science Products, Boston, MA, USA) in 20 μl total volume. The RNA, primer and RNasin were denatured at 75°C for 5 min and cooled on ice before adding the remaining reaction components. After 2 h incubation at 37°C, the heteroduplexes were purified as described for recovery [12], denatured and the mRNA was alkali hydrolyzed for 15 min at 37°C and neutralized with 3 M NaOAc (pH 5.0). The labeled cDNA was purified with a PCR purification kit (Macherey-Nagel) according to the manufacturer's instructions. Probes generated from the control and ischemic or preconditioned heart samples were mixed, reconstituted in 12 μl hybridization buffer (50% formamide, 5×SSC, 0.1% SDS, 100 μg/ml salmon sperm DNA) and applied onto the array after denaturation by heating for 1 min at 90°C. The slide was covered with a 22 mm×22 mm coverslip, and sealed with DPX Mountant (Fluka, Buchs, Switzerland) in order to prevent evaporation. Slides were incubated at 42°C for 20 h in a humid hybridization chamber. After hybridization the mountant was removed and the arrays were washed by submersion and agitation for 10 min in 1×SSC with 0.1% SDS, for 10 min in 0.1%×SSC with 0.1% SDS and for 10 min in 0.1×SSC at room temperature, then rinsed briefly in deionized water and dried.

2.4. Scanning and data analysis

Each array was scanned under a green laser (532 nm) (for Cy3 labeling) and under a red laser (660 nm) (for Cy5 labeling) using a ScanArray Lite (GSI Lumonics, Billerica, MA, USA) scanning confocal fluorescent scanner with 10 μm resolution. Image analysis was performed by ScanAlyze2 software (http://www.microarrays.org/software.html). Each spot was defined by manual positioning of a grid of circles over the image. The average pixel intensity and the local background of each spot were determined. A measure, i.e. 'expression ratio' (MRAT, denotes the median of the set of background-corrected single pixel intensity ratios of the two channels within the spot), was determined [13]. This average expression ratio for all genes on the array was normalized to 1.0. For background corrections those data were calculated as negatives where the average intensity of the spot was smaller than two times the average background of the same area. Significant spots have more than 0.55 CHGTB2 values in both Cy3 and Cy5 channels. Each experiment was performed twice using both fluorescent dyes for labeling control and sample to reduce the number of false positive or false negative ratios deriving from possible uneven incorporation of fluorescent dyes during labeling, or from other experimental variables introduced by hybridization, washing conditions or array features. Therefore, from each RNA pool two probes were generated: a Cy5-labeled and a Cy3-labeled one in order to perform replicate 'color-flip' experiments suggested by other authors [14,15]. Replica spots (on the same array) and replica experiments (two different arrays) resulted in four data points for every gene. Those spots were excluded from further analysis when ratios of the replica spots had a more than two-fold difference. The same restriction was applied for the average ratios of the replica experiments.

2.5. Real-time quantitative PCR

Relative QRT-PCR was performed on a RotorGene 2000 instrument (Corbett Research, Sydney, Australia) with gene-specific primers and SybrGreen protocol to confirm the gene expression changes observed by using microarrays. 20 μg of total RNA from each pool was reverse transcribed in the presence of poly(dT) sequences in a total volume of 20 μl. After dilution of the mix with 80 μl of water, 2 μl of this mix was used as template in the QRT-PCR. Relative expression ratios were normalized to β actin. The PCR primers used in this study are listed in Table 1. All the PCRs were performed in triplicate.

Table 2
Genes with altered expression in response to ischemia/reperfusion when compared to non-ischemic hearts

Functional cluster	Gene product	Accession number	Ratio	S.D.
Bioactive peptides	Atrial natriuretic factor	M27498	2.68	0.45
Cytoskeleton, extracellular matrix proteins	Procollagen, type III, α1	W89883	2.64	0.38
	α-Tubulin	NM_022298	2.66	0.98
Energy metabolism	*Mus domesticus* strain MilP mitochondrion genome	AW545415	0.52	0.1
	NADH-ubiquinone oxidoreductase B15	NM_012985	2.28	0.02
Heat shock proteins	Chaperonin subunit ε	AA956164	1.87	0.11
	Wagneri gene for 105-kDa heat shock protein	AW544862	2.47	0.24
	Heat shock protein, 86 kDa 1	AJ428213	3.5	0.5
Metabolic enzymes	Glycine-*N*-acyltransferase	AA237628	0.57	0.03
	Isocitrate dehydrogenase 3 (NAD^+) α	NM_053638	1.87	0.38
	Formiminotransferase cyclodeaminase	NM_053567	0.5	0.05
	Aconitase 1	AA875134	0.58	0.1
Others	Putative sialoglycoprotease type 2	AA273893	0.56	0.06
	Mouse mRNA for AF1q	AB083464	0.6	0.01
	Elongation factor Tu	AA819168	0.6	0.05
	14-3-3 protein γ subtype	D17447	1.94	0.06
	Homo sapiens SPG protein	AA067045	2.12	0.07
	Mus musculus antigen 4F2	AW545809	2.31	0.07
	Mouse histocompatibility 2, class II antigen A α	NM_010378	0.58	0.02
	GS4	AF492385	1.91	0.03
	Homo sapiens CHMP1.5 protein (CHMP1.5)	AA066250	2.46	0.16
	Signal peptidase	NM_031723	2.8	0.34
	Homo sapiens HSPC245	AA260293	2.01	0.15
	Glutaminyl-tRNA synthetase	BE329136	1.99	0.26
Receptors, ion channels, membrane proteins	Cytokine receptor-like molecule	AA899612	0.59	0.06
	Low-density lipoprotein receptor-related protein	NM_053541	0.6	0.01
	Homo sapiens solute carrier family 39 (zinc transporter)	NM_130849	0.45	0.04
Regulatory proteins, kinases, phosphatases	*Homo sapiens* zinc finger protein 28	AW536277	2.03	0.18
	Fas-activated serine/threonine FAST kinase	AA956496	1.93	0.19
	Mus musculus forkhead protein FKHR	AA221124	2.11	0.21
Signal transduction	Ras oncogene neuroblastoma, Nras	NM_080766	0.59	0.12
Synaptic proteins	Synaptic vesicle protein 2B	AF372834	0.44	0.16
Ubiquitin system	Non-canonical ubiquitin conjugating enzyme 1	AA250689	0.6	0.01
	Rattus norvegicus ubiquitin-like protein	AW545652	2.45	0.39
Unknown	EST	AA276424	0.55	0.11
	Human DNA sequence from clone 1178H5	AW541466	0.58	0.04
	Homo sapiens 12q15 BAC RPCI11-444B24	AW545696	0.58	0.14
	EST	AA261708	0.59	0.04
	EST	AA268104	0.59	0.01
	Clone: 4933424L15: β-defensin-containing protein	NM_031810	0.6	0.06
	EST	AA388512	1.99	0.14
	Mus musculus clone: 2510040L10	AA244814	2.04	0.21
	EST	AA242702	2.07	0.17
	Homo sapiens unknown protein IT12	AW536219	2.18	
	EST	AA412944	2.32	0.14
	EST	AA073606	2.37	0.24
	EST	AA266972	2.49	0.49
	Drosophila melanogaster genomic sequence	AA407331	2.91	0.98

All experiments were done in duplicate and data were calculated from four intensity ratios. 'EST' denotes expressed sequence tag.

3. Results and discussion

3.1. Gene expression alteration after ischemia/reperfusion

Relative gene expression changes in response to ischemia and reperfusion were determined using the expression profiles of time-matched control hearts as baseline. Changes of 3200 genes were followed by rat-specific cDNA microarrays. In response to ischemia and reperfusion, out of 3200 genes 1468 showed significant intensity (see Section 2 for statistical calculations) and 1.6% showed altered expression: 28 genes exhibited significant up-regulation and 21 were down-regulated (Table 2). Little is known about the possible role of most of these genes in ischemia/reperfusion.

In a recent report, global expression analysis in response to renal ischemia was performed by Yoshida et al. [16]. They found that most of the genes showing altered expression are involved in cell structure, extracellular matrix, tissue repair, and cell division/differentiation. By using an Affymetrix oligonucleotide microarray containing 10 000 gene-specific samples they found 122 genes, the expression of which changed due to ischemia-induced acute renal failure. In our present study, several genes with similar characteristics were altered due to myocardial ischemia, i.e. tubulin, procollagen, glycine-*N*-acyltransferase, several metabolic enzymes and proteins involved in programmed cell death. We detected extensive changes in heat shock proteins in our present study. A chaperonin and two heat shock proteins (86 and 105 kDa) were induced by ischemia. The induction of heat stress proteins is well known in response to myocardial, renal, and cerebral ischemia [17–20], however, this is the first demonstration that chaperonin subunit ε is significantly up-regulated due to cardiac ischemia/reperfusion.

Ischemia/reperfusion repressed several genes including some mitochondrial genes and aconitase, a major enzyme of the

Table 3
Genes with altered expression due to preconditioning followed by ischemia/reperfusion when compared to either ischemic/reperfused hearts without preconditioning or non-ischemic controls

Functional cluster	Gene product	Accession number	Ratio 1 (Isc/Norm)	S.D. (1)	Ratio 2 (Prec/Isc)	S.D. (2)
Bioactive peptides	Natriuretic peptide precursor type B	NM_031545	0.73	0.06	1.76	0.21
Calcium-dependent binding proteins	Pentaxin-related gene	W42321	0.97	0.09	1.80	0.24
Cytoskeleton, extracellular matrix proteins	Class I β-tubulin	AB011679	0.65	0.18	0.66	0.01
Heat shock proteins	Chaperonin subunit 5, ε	AA955792	1.87	0.11	2.18	0.21
Metabolic enzymes	(2′,5′)Oligoadenylate synthase 1	Z18877	1.17	0.15	1.75	0.14
	cGMP phosphodiesterase (PDE9A1)	AA273765	1.51	0.26	3.29	0.89
	Peroxisome proliferator activator receptor γ	NM_013124	0.95	0.23	0.50	0.14
	Protease (macropain) 28 subunit, α	NM_017278	1.1	0.31	0.56	0.13
	Betaine-homocysteine methyltransferase	NM_030850	1.36	0.21	0.57	0.05
	N-Acylsphingosine amidohydrolase 1	NM_053407	1.21	0.19	1.70	0.22
Metal binding proteins	Metallothionein II	H32024	1.08	0.16	1.69	0.08
Others	18S, 5.8S, and 28S ribosomal RNAs	V01270	2.8	0.29	1.85	0.14
	Coagulation factor VII	AA271041	1.29	0.2	1.99	0.21
	Nucleolar phosphoprotein of 140 kDa	AA408077	1.07	0.19	2.03	0.31
	Heparin cofactor II	AF096869	1.08	0.09	2.08	0.42
	β-Globin	X05080	1.08	0.34	0.66	0.01
	Kell blood group glycoprotein	AA900226	0.86	0.11	0.51	0.18
	Cysteine proteinase inhibitor	M92418	0.98	0.09	0.63	0.11
Receptors, ion channels, membrane proteins	Secretory carrier membrane protein (SCAMP3)	AF005036	1.78	0.27	1.59	0.05
	Neutral and basic amino acid transporter	U10110	1.78	0.38	1.64	0.14
	Anion exchanger 2	NM_017048	1.76	0.29	1.84	0.28
	Lymphatic endothelium-specific hyaluronan receptor Lyve-1	AA269330	1.88	0.11	0.58	0.01
Regulatory proteins, kinases, phosphatases	Myosin light chain kinase	AW142114	1.02	0.24	0.59	0.14
Signal transduction	Frizzled homolog 4	AW140615	1.2	0.09	0.55	0.19
Unknown	Unknown protein	AA259369	1.09	0.08	3.11	0.64
	Unknown protein	W34106	1.42	0.18	0.47	0.02
	Unknown protein	AA260880	0.92	0.25	0.54	0.21
	Unknown protein	AW539764	1.03	0.06	0.55	0.08
	Unknown protein	AA259659	1.42	0.25	0.57	0.04
	Unknown protein	AA277040	0.78	0.13	0.59	0.14
	Unknown protein		1.27	0.11	0.62	0.09

All experiments were done in duplicate and data were calculated from four intensity ratios. 'Isc' denotes ischemia/reperfusion, 'Norm' non-ischemic controls, and 'Prec' preconditioning.

citrate cycle. It is well known that ischemia/reperfusion results in mitochondrial damage leading to cell apoptosis or necrosis [21–23], however, little is known about the cellular mechanisms of these phenomena.

Lynn et al. studied the gene expression profile of ischemic injury produced by left coronary artery occlusion without reperfusion in mouse hearts. They used an array with only 588 gene-specific probes and found only a small number of genes affected by ischemia. Genes with altered expression were those encoding proteins implicated in oxidative stress, apoptosis and cardiac muscle development [6]. In a rat infarction model, a detailed gene expression analysis was performed using a microarray containing 7000 cDNAs [7] and several genes encoding proteins involved in cytoskeletal architecture, contractility, and metabolism were identified. In accordance with their findings, we found several genes in the present study which exhibited changes in expression in response to ischemia/reperfusion, i.e. heat shock proteins, ubiquinone oxidoreductase, ubiquinone binding protein, collagen, tubulin, and atrial natriuretic factor.

In our present study, several clones encoding hypothetical proteins or ESTs having no homology to known proteins exhibited significant up- or down-regulation. The cellular function of these genes and their relationship to myocardial ischemia/reperfusion needs to be elucidated.

3.2. Gene expression alteration due to preconditioning

To study the effects of preceding preconditioning on ischemia/reperfusion-induced gene expression patterns, we used cDNA microarrays to monitor alterations in gene expression of ischemic rat hearts with and without preconditioning. Genes exhibiting characteristic changes in expression due to preconditioning are shown in Table 3. Out of 3200 rat genes 1450 had significant intensity values but only 1% of them showed altered expression: 14 clones were overexpressed and 17 repressed (see Section 2 for statistical calculations).

In order to confirm the differential expression of genes revealed by microarray analysis of rat hearts after ischemia with and without preconditioning, several genes were analyzed by real-time fluorescent QRT-PCR. We selected seven genes of which the expression was significantly altered in preconditioned hearts for real-time RT-PCR analysis (Fig. 1). The differential expression of these genes revealed an almost perfect concordance with the microarray data. Genes encoding chaperonin subunit ε, anion exchange protein 2 and metallothionein II had a very significant rise in transcription rate,

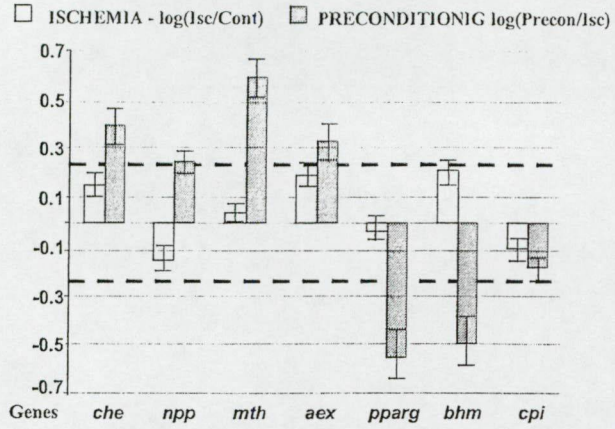


Fig. 1. Quantitative determination of transcript levels by real-time PCR. Changes in transcript levels in rat hearts during ischemia (light box) and ischemia with preconditioning (dark box) were confirmed by triplicate real-time PCR. β-Actin was used as a control. The expression of the following genes was determined: chaperonin subunit ε: *che*; natriuretic peptide precursor type B: *npp*; anion exchange protein 2: *aex*; metallothionein II: *mth*; peroxisome proliferator activator receptor γ: *pparg*; betaine-homocysteine methyltransferase: *bmh*; cysteine proteinase inhibitor: *cpi*. Dashed lines indicate the interval −1.8- to 1.8-fold regulation (corresponding to $\log_{10} = 0.255$) in which changes in expression were considered not significant.

while the natriuretic peptide precursor type B gene showed a less pronounced induction. Genes encoding peroxisome proliferator activator receptor γ (PPARγ) and betaine-homocysteine methyltransferase showed repression, although the cysteine proteinase inhibitor gene exhibited moderate repression at the mRNA level.

Changes in the expression of some genes by preconditioning followed by ischemia/reperfusion were similar to those changed by ischemia/reperfusion alone (overexpressed: a secretory membrane protein, an amino acid transporter, an anion exchanger, a ribosomal RNA and a chaperonin gene; repressed: β-tubulin). Because in the case of preconditioning the control sample was ischemia/reperfusion alone, the expression of these genes changed more dramatically when compared to non-ischemic controls. This suggests that these genes might have significant roles in ischemic adaptation of the heart during single ischemia without preconditioning as well. The rest of the genes listed in Table 3 are those which were specifically and differentially expressed in response to preconditioning and were not altered after single ischemia/reperfusion. Among these genes metallothionein, coagulation factor VII, cysteine proteinase inhibitor, PPARγ and myosin light chain kinase genes were previously shown to have connections with ischemia or other heart diseases [24–33]. Hypoxia preconditioning induced the expression of metallothionein in the brain [25]. We found here that the (2′,5′)oligoadenylate synthase gene showed overexpression in response to preconditioning. It has been previously shown by others that the mRNA level of this gene rose more than two- to three-fold after 24 h recovery from ischemia in the rat brain [26]. Therefore, it is plausible to speculate that oligoadenylate synthase might have a protective effect on the heart as well. Chaperonin subunit ε and natriuretic peptide precursor type B also exhibited up-regulation due to preconditioning. None of these genes has previously been shown to be involved in preconditioning.

A more dramatic induction was detected in the expression of a cGMP phosphodiesterase (PDE9A1). Alterations in cGMP levels in the heart have previously been shown in response to preconditioning [27], however, this is the first demonstration that the expression of a phosphodiesterase gene is altered due to preconditioning.

PPARγ exhibited one of the most pronounced repressions due to preconditioning. PPARγ has been shown to be involved in several cardiovascular pathologies including atherosclerosis and ischemic heart disease, however, this is the first demonstration that PPARγ plays a role in ischemic preconditioning. Interestingly, most of the previous studies show that pharmacological activation of PPARγ protects the ischemic heart [28–30]. In contrast, our present study shows that preconditioning leads to a marked repression of the PPARγ gene. This suggests that the role of PPARγ in ischemic injury and ischemic adaptation is still unclear.

Degradation of myocardial structural proteins in myocardial infarction has been shown to be reduced by a cysteine proteinase inhibitor [31]. In our present study a 1.59-fold repression was detected by microarray analysis and a 1.75-fold repression by real-time quantitative PCR. It seems that the activity of cysteine proteases is favored in preconditioning. Another gene related to protein degradation was also repressed: protease 28 subunit had a 1.79-fold repression; this gene has a regulatory function in proteasome for small protein

Table 4
Heart rate, coronary flow, and lactate dehydrogenase (LDH) release in non-ischemic time-matched control, ischemia/reperfusion, and preconditioning+ischemia/reperfusion groups at the beginning of perfusion, upon early reperfusion (rep.) and at the end of the reperfusion in isolated rat hearts

Group	Basal	5 min rep.	120 min rep.
Heart rate (beats/min)			
Non-ischemic control	244 ± 4	243 ± 5	244 ± 5
Ischemia/reperfusion	240 ± 4	246 ± 12	249 ± 9
Preconditioning+ischemia/reperfusion	248 ± 6	248 ± 12	246 ± 7
Coronary flow (ml/min/g)			
Non-ischemic control	14.7 ± 1.0	17.0 ± 1.0	17.0 ± 0.9
Ischemia/reperfusion	15.8 ± 1.1	15.7 ± 2.9	12.2 ± 1.2*
Preconditioning+ischemia/reperfusion	15.6 ± 1.2	16.3 ± 1.6	15.6 ± 0.9
LDH release (mU/min/g)			
Non-ischemic control	nd	nd	nd
Ischemia/reperfusion	nd	671 ± 98*	422 ± 72*
Preconditioning+ischemia/reperfusion	nd	148 ± 56*#	223 ± 57*

Data are means ± S.E.M.; nd, non-detectable, below the detection limit ($n = 5$–8 in each group).
*$P < 0.05$ vs. corresponding control, #$P < 0.05$ vs. ischemia/reperfusion.

substrate degradation [32] and has implications for oxidative stress [33].

3.3. Limitations of the study

The stability of the preparation upon long-term perfusion is a general concern in isolated heart preparations. Here we used a Langendorff preparation with no left ventricular balloon to unload the heart from 'afterload pressure' and to maintain a good coronary perfusion throughout the perfusion protocol. As shown in Table 4, in the non-ischemic time-matched control group, heart rate and coronary flow were stable and no LDH release was detected which show the stable aerobic condition of the heart. Preconditioning significantly decreased postischemic LDH release showing the well-known cardioprotective effect of preconditioning in this model (Table 4). As the present study was performed in crystalloid-perfused isolated rat hearts and the analysis of gene expression was done using cardiac tissue that did not contain components of blood, the mechanisms of ischemia/reperfusion injury and preconditioning might be somewhat different in the present ex vivo experimental model as compared to in vivo situations. A further limitation of the present study is that analysis of cardiac tissue for gene expression pattern was done at the end of the 2-h reperfusion to allow time for mRNA accumulation and/or degradation. Therefore, the present study cannot distinguish between the 'trigger' and 'mediator' genes of preconditioning (see [2–4] for reviews); however, addressing this issue raises many technical problems in study design and needs further studies in the future.

In summary, here we present a number of genes of which the expression is significantly altered due to ischemia/reperfusion and preconditioning in the heart. Although some of the genes have previously been shown to play a role in ischemia/reperfusion injury, this is the first demonstration that most of the genes presented here are involved in classic preconditioning and ischemia/reperfusion. The newly identified genes in our present study might lead to a better understanding of the cellular mechanisms of the remarkable cardioprotection elicited by ischemic preconditioning.

Acknowledgements: This work was supported by grants from the Hungarian Scientific Research Foundation (OTKA T029843), Hungarian Ministry of Education (FKFP-0340/2000 and FKFP-0057/2001) and Ministry of Health (ETT 51/2000), and the Hungarian National R&D Program (NFKP-1/040).

References

[1] Przyklenk, K. and Kloner, R.A. (1998) Prog. Cardiovasc. Dis. 40, 517–547.
[2] Baxter, G.F. and Ferdinandy, P. (2001) Basic Res. Cardiol. 96, 329–344.
[3] Ferdinandy, P., Szilvassy, Z. and Baxter, G.F. (1998) Trends Pharmacol. Sci. 19, 223–229.
[4] Bolli, R., Dawn, B., Tang, X.L., Qiu, Y., Ping, P., Xuan, Y.T., Jones, W.K., Takano, H., Guo, Y. and Zhang, J. (1998) Basic Res. Cardiol. 93, 325–338.
[5] Schulz, R., Cohen, M.V., Behrends, M., Downey, J.M. and Heusch, G. (2001) Cardiovasc. Res. 52, 181–198.
[6] Lyn, D., Liu, X., Bennett, N.A. and Emmett, N.L. (2000) Physiol. Genomics 2, 93–100.
[7] Stanton, L.W., Garrard, L.J., Damm, D., Garrick, B.L., Lam, A., Kapoun, A.M., Zheng, Q., Protter, A.A., Schreiner, G.F. and White, R.T. (2000) Circ. Res. 86, 939–945.
[8] Csonka, C., Szilvassy, Z., Pali, T., Blasig, I.E., Tosaki, A., Schulz, R. and Ferdinandy, P. (1999) Circulation 100, 2260–2266.
[9] Csonka, C., Csont, T., Onody, A. and Ferdinandy, P. (2001) Biochem. Biophys. Res. Commun. 285, 1217–1219.
[10] Kitajka, K., Puskas, L.G., Zvara, A., Hackler, L.Jr., Barcelo-Coblijn, G., Yeo, Y.K. and Farkas, T. (2002) Proc. Natl. Acad. Sci. USA 99, 2619–2624.
[11] Puskas, L.G., Zvara, A., Hackler, L.Jr. and van Hummelen, P. (2002) BioTechniques 32, 1330–1340.
[12] Puskas, L.G., Hackler Jr., L., Kovacs, G., Kupihar, Z., Zvara, A., Micsik, T. and van Hummelen, P. (2002) Anal. Biochem. 305, 279–281.
[13] Eisen, M.B., Spellman, P.T., Brown, P.O. and Botstein, D. (1998) Proc. Natl. Acad. Sci. USA 95, 1483–1488.
[14] Lee, M-L.T., Kuo, F.C., Whitemore, G.A. and Sklar, J. (2000) Proc. Natl. Acad. Sci. USA 97, 9834–9839.
[15] Stuart, R.O., Bush, K.T. and Nigman, S.K. (2001) Proc. Natl. Acad. Sci. USA 98, 5649–5654.
[16] Yoshida, T., Tang, S.S., Hsiao, L.L., Jensen, R.V., Ingelfinger, J.R. and Gullans, S.R. (2002) Biochem. Biophys. Res. Commun. 291, 787–794.
[17] Kelly, K.J. (2002) Minerva Urol. Nefrol. 54, 81–91.
[18] Snoeckx, L.H., Cornelussen, R.N., Van Nieuwenhoven, F.A., Reneman, R.S. and Van Der Vusse, G.J. (2001) Physiol. Rev. 81, 1461–1497.
[19] Kitagawa, K., Matsumoto, M. and Hori, M. (2001) Can. J. Physiol. Pharmacol. 79, 262–265.
[20] Papadopoulos, M.C., Giffard, R.G. and Bell, B.A. (2000) Br. J. Neurosurg. 14, 305–312.
[21] Lust, W.D., Taylor, C., Pundik, S., Selman, W.R. and Ratcheson, R.A. (2002) Metab. Brain Dis. 17, 113–121.
[22] Scarabelli, T.M., Stephanou, A., Pasini, E., Comini, L., Raddino, R., Knight, R.A. and Latchman, D.S. (2002) Circ. Res. 90, 745–748.
[23] Jassem, W., Fuggle, S.V., Rela, M., Koo, D.D. and Heaton, N.D. (2002) Transplantation 73, 493–499.
[24] Okawa, H., Horimoto, H., Mieno, S., Nomura, Y., Nakahara, K., Yoshida, M., Asada, K. and Sasaki, S. (2002) J. Cardiol. 39, 299–304.
[25] Emerson, M.R., Samson, F.E. and Pazdernik, T.L. (2000) Cell. Mol. Biol. (Noisy-le-Grand) 46, 619–626.
[26] Paschen, W., Althausen, S. and Doutheil, J. (1999) Neurosci. Lett. 263, 109–112.
[27] Szilvassy, Z., Ferdinandy, P., Bor, P., Jakab, I., Lonovics, J. and Koltai, M. (1994) Am. J. Physiol. 266, 2033–2041.
[28] Fruchart, J.C., Duriez, P. and Staels, B. (1999) Curr. Opin. Lipidol. 10, 245–257.
[29] Thiemermann, C. and Wayman, N.S. (2001) Med. Sci. Monit. 7, 787–789.
[30] Fruchart, J.C., Duriez, P. and Staels, B. (1999) Curr. Opin. Lipidol. 10, 245–257.
[31] Tsuchida, K., Aihara, H., Isogai, K., Hanada, K. and Shibata, N. (1986) Biol. Chem. Hoppe-Seyler 367, 39–45.
[32] Ma, C.P., Slaughter, C.A. and DeMartino, G.N. (1992) J. Biol. Chem. 267, 10515–10523.
[33] Keller, J.N., Hanni, K.B. and Markesbery, W.R. (2000) Mech. Ageing Dev. 113, 61–70.

III

ELSEVIER

Cardiovascular Research 58 (2003) 663–670

Cardiovascular Research

www.elsevier.com/locate/cardiores

Hyperlipidemia induced by a cholesterol-rich diet leads to enhanced peroxynitrite formation in rat hearts

Annamária Ónody, Csaba Csonka, Zoltán Giricz, Péter Ferdinandy*

Cardiovascular Research Group, Department of Biochemistry, Faculty of Medicine, University of Szeged, Dóm tér 9, H-6720 Szeged, Hungary

Received 17 February 2003; accepted 20 February 2003

Abstract

Objective: We investigated the influence of experimental hyperlipidemia on the formation of cardiac NO, superoxide, and peroxynitrite ($ONOO^-$) in rat hearts. **Methods:** Wistar rats were fed 2% cholesterol-enriched diet or normal diet for 8 weeks. Separate groups of normal and hyperlipidemic rats were injected twice intraperitoneally with 2×20 μmol/kg FeTPPS (5,10,15,20-tetrakis-[4-sulfonatophenyl]-porphyrinato-iron[III]), a $ONOO^-$ decomposition catalyst, 24 h and 1 h before isolation of the hearts. **Results:** A cholesterol diet significantly decreased myocardial NO content, however, myocardial Ca^{2+}-dependent and Ca^{2+}-independent NO synthase activity and NO synthase protein level did not change. Myocardial superoxide formation and xanthine oxidase activity were significantly increased; however, cardiac superoxide dismutase activity did not change in the cholesterol-fed group. Dityrosine in the perfusate, a marker of cardiac $ONOO^-$ formation, and plasma nitrotyrosine, a marker for systemic $ONOO^-$ formation, were both elevated in hyperlipidemic rats. In cholesterol-fed rats, left ventricular end-diastolic pressure (LVEDP) was significantly elevated as compared to controls. Administration of FeTPPS normalized LVEDP in the cholesterol-fed group. **Conclusion:** We conclude that cholesterol-enriched diet-induced hyperlipidemia leads to an increase in cardiac $ONOO^-$ formation and a decrease in the bioavailability of NO which contributes to the deterioration of cardiac performance and may lead to further cardiac pathologies.

Keywords: Cholesterol; Contractile function; Enzyme; Free radicals; Nitric oxide

1. Introduction

High-cholesterol diet is regarded as an important factor in the development of cardiac diseases since it leads to development of hyperlipidemia, atherosclerosis, and ischemic heart disease. The heart of hyperlipidemic/atherosclerotic patients adapts poorly to oxidative or other kinds of stress, suggesting that the endogenous adaptive mechanisms against myocardial stress are impaired [1]. The focus of research so far has been mainly on the coronary effects of cholesterol, i.e. coronary sclerosis, and the possible direct effect of hypercholesterolemia on the heart was neglected. Very few studies looked at the cellular effects of cholesterol-enriched diet on the myocardium; however, intracellular lipid accumulation in cardiomyocytes and several alterations in the structural and functional properties of the myocardium have been observed [2,3]. Furthermore, we have previously shown that cholesterol-enriched diet-induced hyperlipidemia attenuates the cardioprotective effect of ischemic preconditioning via a mechanism independent from the vascular effects of hyperlipidemia ([4,5], see Ref. [6] for review).

Increasing evidence accumulated in recent years showing that high-cholesterol diet impairs NO-cGMP signaling in both endothelial and nonendothelial cells [4,7–9]. In the normal heart, nitric oxide (NO) is synthesized by Ca^{2+}-dependent NO synthases in cardiac myocytes, vascular and endocardial endothelium (NO synthase III) as well as in specific cardiac neurons (NO synthase I) and plays an important role in the regulation of coronary circulation and cardiac contractile function [10]. Atherosclerosis is a well known 'NO deficient state' in the vasculature which leads

*Corresponding author. Tel.: +36-62-545-755; fax: +36-62-545-097.
E-mail address: peter@bioch.szote.u-szeged.hu (P. Ferdinandy), http://www.cardiovasc.com

Time for primary review 28 days.

0008-6363/03/$ – see front matter
doi:10.1016/S0008-6363(03)00330-4

to sustained arterial hypertension (see Ref. [11] for review) and reduced cardiovascular tolerance to stress (see Ref. [6] for review). Lefer and Ma [7] observed reduced NO release from rabbit aorta in hypercholesterolemia, and Deliconstantinos et al. [9] showed that incorporation of high concentrations of cholesterol into endothelial cell membranes caused downregulation of NO synthase. Reduced vascular NO release in hyperlipidemia has been also shown as a consequence of increased formation of superoxide, which then reacts with NO to form peroxynitrite ($ONOO^-$) [12,13]. However, the effect of hyperlipidemia on the formation of NO, superoxide, and $ONOO^-$ in the heart is not known.

We have previously shown that cardiac NO level is significantly decreased in hearts of cholesterol-fed rats [4]. However, the mechanism of reduced NO level in the heart is not known. Here we hypothesized, that the decrease in cardiac NO level is secondary to increased production of superoxide and formation of $ONOO^-$. Therefore, in the present study we systematically analyzed if hyperlipidemia influences formation of cardiac NO, superoxide, and $ONOO^-$. Here we measured myocardial levels of NO, superoxide, and their reaction product $ONOO^-$. We also determined activities of major enzymatic sources for NO and superoxide, i.e. NO synthases and xanthine oxidoreductase, the major antioxidant enzyme superoxide dismutase (SOD) as well as several parameters of myocardial contractile function.

2. Methods

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the ethics committee of the University of Szeged.

2.1. Experimental groups, induction of hyperlipidemia

Male Wistar rats (18-weeks-old), housed in a room maintained at 12 h light–dark cycles and a constant temperature of 22±2 °C, were fed laboratory chow enriched with 2% cholesterol or standard chow for 8 weeks. At the end of the diet period, hearts were isolated for measurement of cardiac function and biochemical parameters. Separate groups of normal and hyperlipidemic rats were injected twice intraperitoneally with 2×20 μmol/kg FeTPPS (5,10,15,20-tetrakis-[4-sulfonatophenyl]-porphyrinato-iron[III]), a $ONOO^-$ decomposition catalyst, 24 h and 1 h before the isolation of the hearts to allow sufficient time for repair mechanisms and de novo protein synthesis to recover $ONOO^-$-induced cellular injury. At the end of the 8-week diet period, body weights of the animals were 350–400 g, and there were no significant difference between groups; plasma cholesterol and triglyceride level increased by 20% and 300%, respectively, which was consistent with our previous findings [14].

2.2. Measurement of cardiac function in isolated rat hearts

At the end of the diet and FeTPPS treatment, rats were anesthetized with diethyl ether and injected intravenously with 500 U/kg heparin. After 30 s, hearts were excised and placed in perfusion fluid of 4 °C until contractions ceased. Each heart was then cannulated through the aorta and the left atrium and prepared for working heart perfused at 37 °C with Krebs–Henseleit bicarbonate buffer containing (in mM) NaCl 118.4, KCl 4.1, $CaCl_2$ 2.5, $NaHCO_3$ 25, KH_2PO_4 1.17, $MgCl_2$ 1.46 and glucose 11.1; gassed with 95% O_2 and 5% CO_2 and supplemented with 0.3 mmol/l L-tyrosine [15]. Preload (1.7 kPa) and afterload (9.8 kPa) were kept constant throughout the experiments. After a 10 min normoxic, normothermic perfusion, cardiac mechanical functional and hemodynamic parameters including heart rate (HR), coronary flow (CF), aortic flow (AF), left ventricular developed pressure (LVDP) and its first derivatives ($+dP/dt_{max}$, $-dP/dt_{max}$), and left ventricular end-diastolic pressure (LVEDP) were monitored as described [15,16]. Coronary effluent and myocardial tissue were sampled and frozen in liquid nitrogen for further biochemical measurements.

2.3. Measurement of cardiac NO and superoxide

In separate experiments, NO content of ventricular tissue was measured using electron spin resonance spectroscopy after loading the heart with the NO-specific spin trap Fe^{2+}-*N*-methyl-D-glucosamine-dithiocarbamate (MGD) as described [15,16]. The spin-trap for NO was prepared freshly before each experiment. MGD (175 mg) and $FeSO_4$ (50 mg) dissolved in distilled water (pH 7.4, volume 6 ml) was infused into the aortic cannula under Langendorff perfusion (constant pressure at 9.8 kPa) for 5 min at a rate of 1 ml/min in order to measure basal myocardial NO content. Tissue samples from the apex of the heart (approximately 150 mg) were collected at the end of the infusion of $Fe^{2+}(MGD)_2$ and placed into quartz ESR tubes and frozen in liquid nitrogen. Electron spin resonance spectra of $NO–Fe^{2+}(MGD)_2$ adducts were recorded with a Bruker ECS106 spectrometer (Rheinstetten, Germany; ESR parameters: X band, 100 kHz modulation frequency, 160 K temperature, 10 mW microwave power, 2.85 G modulation amplitude, 3356 G central field) and analyzed for NO signal intensity as described [16].

Superoxide production in freshly minced ventricles was assessed by lucigenin-enhanced chemiluminescence [15]. Approximately 100 mg of the apex of the heart was placed in 1 ml air-equilibrated Krebs–Henseleit solution containing 10 mmol/l HEPES–NaOH (pH 7.4) and 5 μmol/l

lucigenin. Chemiluminescence was measured at room temperature in a liquid scintillation counter using a single active photomultiplier positioned in out-of-coincidence mode in the presence or absence of the superoxide scavenger nitroblue tetrazolium (NBT, 200 μmol/l). NBT-inhibitable chemiluminescence was considered an index of myocardial superoxide generation. It should be noted that NBT, like other superoxide scavengers, is not entirely specific for superoxide.

2.4. Measurement of cardiac NO synthase, xanthine oxidoreductase, and SOD activities

To estimate endogenous enzymatic NO production, Ca^{2+}-dependent and Ca^{2+}-independent NO synthase activities in ventricular homogenates were measured by the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline as we previously described [15]. Powdered frozen ventricular tissue was placed in four volumes of ice-cold homogenization buffer (composition given in Ref. [17]) and homogenized with an Ultra-Turrex disperser. The homogenate was centrifuged (1000×*g* for 10 min) at 4 °C and the supernatant was kept on ice for immediate assay of enzyme activities. The protein concentration was measured from the supernatant using a Lowry–Folin method. Samples were incubated for 25 min at 37 °C in the presence or absence of either EGTA (1 mM) or EGTA plus N^G-monomethyl-L-arginine (1 mM) to determine the level of Ca^{2+}-dependent and Ca^{2+}-independent NO synthase activities, respectively.

Activity of xanthine oxidoreductase (xanthine oxidase and xanthine dehydrogenase), one of the dominant sources of superoxide in rat hearts, was determined from ventricular homogenates by a fluorometric kinetic assay based on the conversion of pterine to isoxanthopterine in the presence (total xanthine oxidoreductase activity) and absence (xanthine oxidase activity) of the electron acceptor methylene blue as described [15,18]. Ventricular homogenates were prepared as for the measurement of NO synthase activity.

Total activity of SOD was measured by a spectrophotometric assay using a kit (Randox Laboratories Ltd, UK). Approximately 100 mg ventricular tissue was homogenized in 10 volumes of ice-cold phosphate buffer (0.01 M, pH 7.0). Total SOD activity in homogenates was determined by the inhibition of formazan dye formation due to superoxide generated by xanthine and xanthine oxidase.

2.5. Measurement of cardiac NO synthase III (Western blot)

Ventricular homogenates used for NO synthase activity assays were further diluted in the homogenization buffer to allow loading of 25 μg of total protein in each lane of 8% polyacrylamide gel. Electrophoresis was conducted at 200 V, 8 mA for 1.5 h, and proteins were transferred on to nitrocellulose membrane (25 V, 200 mA, 4 °C, 1.5 h) by Western blotting. Membranes were then incubated at room temperature for 1 h with monoclonal anti-NO synthase III antibody (Transduction Laboratories Lexington, KY, USA) at 1:200 dilution, and then with horseradish peroxidase-conjugated polyclonal goat anti-mouse IgG (Transduction Laboratories) at 1:500 dilution for 2 h. The membranes were then developed with an enhanced chemiluminescence kit (NEN Boston, MA, USA). NO synthase III level was assessed by densitometry.

2.6. Measurement of markers of $ONOO^-$

We measured both dityrosine by spectrofluorometry and free nitrotyrosine by enyme-linked immunosorbent assay (ELISA; Cayman Chemical, Ann Arbor, MI, USA) in the perfusate as markers of cardiac $ONOO^-$ formation [15]. $ONOO^-$ promotes nitration of phenolic compounds such as tyrosine, the nitration of which leads to the formation of stable products, dityrosine and 3-nitrotyrosine. Therefore, to measure cardiac $ONOO^-$ generation, Krebs–Henseleit buffer was supplemented with L-tyrosine and dityrosine and nitrotyrosine formation was detected in the coronary effluent as described [15]. Dityrosine and nitrotyrosine formation was normalized to coronary flow and wet weight of the hearts and expressed as pmoles/min/mg protein.

We also measured plasma nitrotyrosine as a marker of systemic $ONOO^-$ generation as described [13]. Plasma $ONOO^-$ concentration was expressed as nmoles/l.

2.7. Measurement of plasma malondialdehyde (MDA)

MDA is a marker of lipid peroxidation that reacts with thiobarbituric acid (TBA). As the reaction is not entirely specific for MDA, the assay is called the thiobarbituric acid-reactive substance (TBARS) assay [19]. Plasma samples were mixed thoroughly with 1.2 volumes of a stock solution of 15% w/v trichloroacetic acid, 0.375% w/v TBA and 0.25 N HCl and heated for 30 min at 95 °C. After cooling and centrifugation at 1000×*g* for 10 min, the supernatant containing TBARS were extracted in butanol and assayed spectrophotometrically at 535 nm. Freshly diluted tetramethoxypropane which yields MDA was used as the external standard.

2.8. Statistical analysis

Data were expressed as means±S.E.M. and analyzed with unpaired *t*-test or ANOVA followed by Tukey's test as appropriate. $P<0.05$ was accepted as a statistically significant difference.

3. Results

3.1. Cardiac NO content and NO synthase

Myocardial NO content was significantly decreased in the hyperlipidemic group as measured by electron spin resonance spectroscopy after ex vivo spin trapping of NO in isolated hearts (Fig. 1A).

To test whether a decrease in cardiac NO in hyperlipidemia is a consequence of diminished enzymatic synthesis, we measured cardiac activities of NO synthases. Endogenous enzymatic sources of NO, Ca^{2+}-dependent and Ca^{2+}-independent NO synthase activities (Fig. 1B) were not changed in the myocardium due to cholesterol diet. As Ca^{2+}-independent NO synthase activity was negligible in both groups, we have measured only NO synthase III protein content. NO synthase III did not change in hyperlipidemic hearts when compared to controls (Fig. 1C).

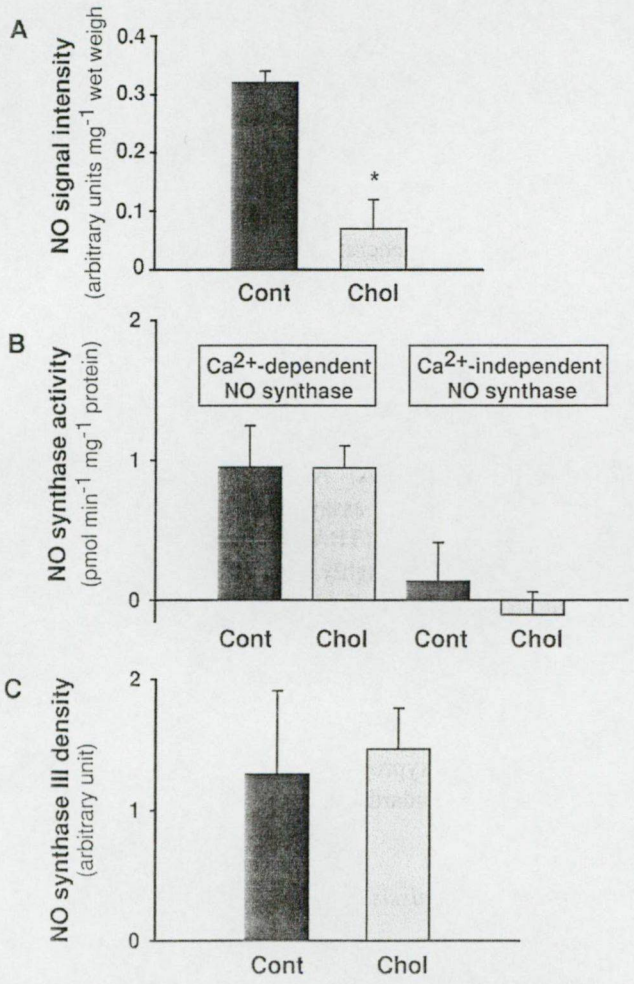


Fig. 1. Myocardial NO content (A), myocardial Ca^{2+}-dependent and Ca^{2+}-independent NO synthase activity (B), and NO synthase III protein content (C) in the control (Cont) and cholesterol-fed (Chol) groups. Results are means±S.E.M. ($n=7$ in both groups). *$P<0.05$ vs. control.

3.2. Cardiac superoxide, xanthine oxidase and superoxide dismutase

To test if cholesterol-enriched diet increases cardiac superoxide generation, we performed a lucigenin-enhanced chemiluminescence assay in freshly minced cardiac tissue. Cardiac superoxide generation was significantly increased due to the high-cholesterol diet as compared to controls (Fig. 2A). To test possible changes in the enzymatic synthesis of superoxide, we measured the activity of xanthine oxidoreductase enzyme complex, one of the major enzymatic sources of superoxide in rat hearts. Xanthine oxidase activity was significantly increased in the hyperlipidemic group (Fig. 2B). We also assayed total activity of SOD in the myocardium, the major enzyme

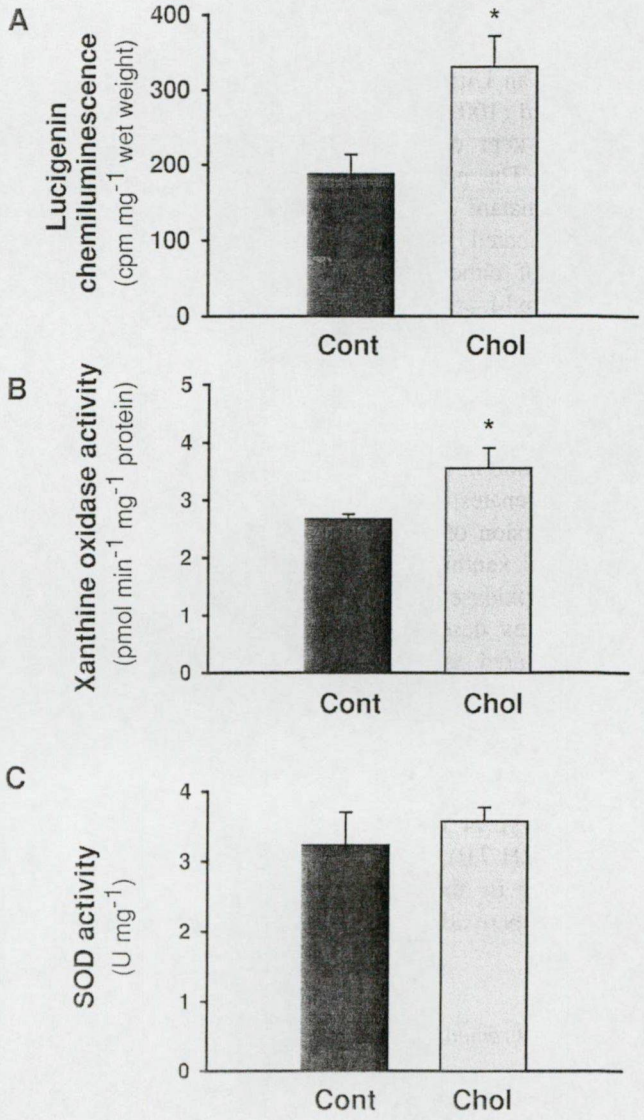


Fig. 2. Cardiac superoxide production (A), myocardial xanthine oxide activity (B), and myocardial SOD activity (C) in the control (Cont) and cholesterol-fed (Chol) groups. Results are means±S.E.M. ($n=7$ in both groups). *$P<0.05$ vs. control.

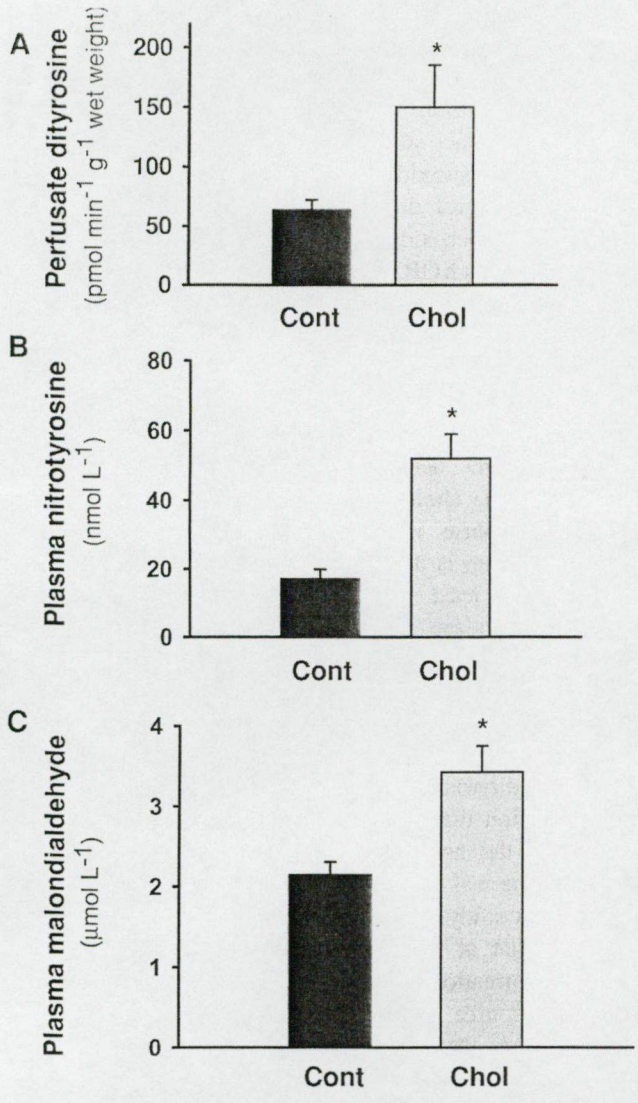


Fig. 3. Dityrosine (A) formation in the perfusate, a marker for cardiac peroxynitrite generation; plasma nitrotyrosine (B) concentration, a marker for systemic peroxynitrite generation; and plasma malondialdehyde (C) concentration, a marker for systemic lipid peroxidation in the control (Cont) and cholesterol-fed (Chol) groups. Results are means±S.E.M. (n=7 in both groups). *P<0.05 vs. control.

responsible for detoxification of superoxide. SOD activity was not changed in hyperlipidemic hearts when compared to controls (Fig. 2C).

3.3. Cardiac $ONOO^-$

To test formation of $ONOO^-$ in the heart, isolated hearts obtained from cholesterol-fed and control groups were perfused with a buffer supplemented with 0.3 mmol/l L-tyrosine. Markers of cardiac $ONOO^-$ generation, dityrosine (Fig. 3A) in the coronary effluent, were increased in the cholesterol-fed group as compared to controls. The formation of the other cardiac peroxynitrite marker, nitrotyrosine, in the coronary effluent, was not statistically significantly increased in the cholesterol-fed group (control 10.48±2.27; cholesterol-fed 14.19±4.56).

3.4. Systemic $ONOO^-$

We also studied if high-cholesterol diet increased systemic formation of $ONOO^-$. Therefore, plasma free nitrotyrosine concentration was measured in control and cholesterol-fed groups as a marker for systemic $ONOO^-$ formation. Plasma free nitrotyrosine was increased approximately two-fold in cholesterol-fed rats as compared to controls (Fig. 3B).

3.5. Plasma malondialdehyde

We also studied if high-cholesterol diet increased systemic lipid peroxidation due to oxidative stress. Therefore, plasma malondialdehyde concentration was measured in control and cholesterol-fed groups as a marker for systemic lipid peroxidation. Plasma malondialdehyde was significantly increased in cholesterol-fed rats as compared to controls (Fig. 3C).

3.6. Cardiac function

To test if an increase in cardiac $ONOO^-$ formation leads to alterations in cardiac performance, cardiac contractile parameters were measured in isolated working hearts. LVEDP was significantly increased in the hyperlipidemic group as compared to controls. Other parameters of cardiac performance such as heart rate, aortic flow, coronary flow, left ventricular developed pressure, $+dP/dt_{max}$, $-dP/dt_{max}$ were not affected significantly by cholesterol-diet when compared to the control group (Table 1). To further test if hyperlipidemia-induced elevation of LVEDP was due to enhanced $ONOO^-$ formation, hyperlipidemic and normal

Table 1
Cardiac functional parameters in the control and cholesterol-fed groups

	HR	CF	AF	CO	LVDP	$+dP/dt_{max}$	$-dP/dt_{min}$	LVEDP
Control	271.0±7.5	22.9±0.5	43.4±2.0	66.3±2.5	18.2±0.4	839.5±45.2	460.0±34.2	0.52±0.05
Cholesterol-fed	270.3±9.4	22.1±0.5	45.3±1.2	67.4±1.6	18.9±0.4	945.0±40.2	483.8±41.6	0.85±0.05*

Heart rate (HR, beats/min); coronary flow (CF, ml/min); aortic flow (AF, ml); cardiac output (CO, ml/min); left ventricular developed pressure (LVDP, kPa); left ventricular end-diastolic pressure (LVEDP, kPa); $+dP/dt_{max}$ (kPa/s); $-dP/dt_{max}$ (kPa/s). Values are means±S.E.M. (n=8 in each group). *P<0.05 shows significant difference compared to control.

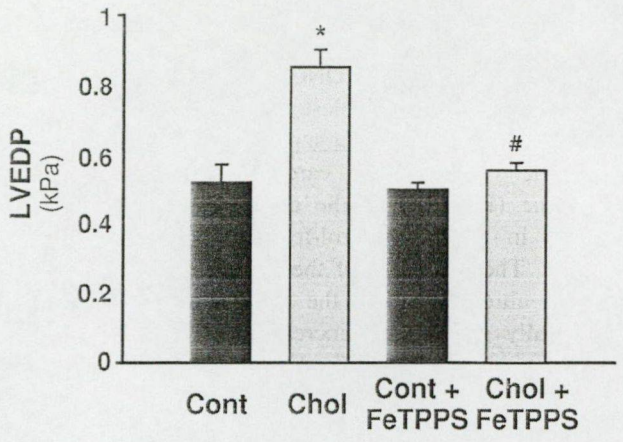


Fig. 4. Left ventricular end-diastolic pressure (LVEDP) in control (Cont), cholesterol-fed (Chol), and cholesterol-fed+FeTPPS-treated (Chol+FeTPPS) groups. Results are means±S.E.M. (n=8 in each group). $^{*}P<0.05$ vs. control, $^{\#}P<0.05$ vs. cholesterol-fed.

rats were treated with FeTPPS, a $ONOO^-$ decomposition catalyst. In the hyperlipidemic group, LVEDP was recovered to control values after FeTPPS treatment, however, FeTPPS did not change LVEDP in the normal group (Fig. 4).

4. Discussion

The present results show that cholesterol-enriched diet for 8 weeks markedly reduces cardiac NO level, enhances cardiac formation of superoxide and their reaction product $ONOO^-$, thereby leading to an increase in LVEDP, which can be prevented by pretreatment with a $ONOO^-$ decomposition catalyst, FeTPPS. This is the first demonstration that high-cholesterol diet leads to enhanced $ONOO^-$ formation in the heart which results in a deterioration of cardiac function.

In accordance with our previous studies [4] we have found in the present study that high-cholesterol diet leads to a decrease in cardiac NO level. Here we further tested if a decrease in cardiac NO is due to diminished NO biosynthesis. Therefore, we measured the activity and the protein content of endogenous enzymatic sources of NO, Ca^{2+}-dependent and Ca^{2+}-independent NO synthases. We have shown here that high-cholesterol diet does not affect the activity of NO synthases and NO synthase III protein content in the myocardium. This finding shows that high-cholesterol diet-induced decrease in cardiac NO is not a consequence of diminished enzymatic synthesis. It was therefore plausible to speculate that cholesterol diet leads to an enhanced elimination of NO.

It is well known that NO rapidly reacts with superoxide to form the cytotoxic species $ONOO^-$ [20]. Hyperlipidemia has been shown to increase production of reactive oxygen species including $ONOO^-$ in the vasculature [13,20–22]. Although it is not known if hyperlipidemia leads to increased formation of reactive oxygen species in the heart, it is plausible to speculate that this mechanism is involved in the enhanced breakdown of NO in the myocardium in hyperlipidemia. Therefore, we measured cardiac superoxide and $ONOO^-$ production. Myocardial superoxide level was significantly increased due to cholesterol diet in this study. One of the major sources of superoxide in the rat heart is the xanthine oxidoreductase (XOR) enzyme [23], which comprises both xanthine dehydrogenase (XDH) and xanthine oxidase (XO). Our results show that hyperlipidemia increased myocardial activity of XO. We also assessed if a diminished elimination of superoxide also plays a role in cholesterol-rich diet-induced enhanced superoxide level, however, SOD activity in the myocardium was not changed in the cholesterol-fed group. These results show that the increase in cardiac superoxide in the hyperlipidemic group is a consequence of increased superoxide synthesis at least in part by enhanced XO activity. NAD(P)H oxidase activity, another important source of cardiac superoxide has not been measured in this study.

In addition to increased myocardial superoxide formation, we have found here that high-cholesterol diet increases formation of a potential marker of cardiac $ONOO^-$, dityrosine in the perfusate. This is the first demonstration that hyperlipidemia increases $ONOO^-$ formation in the heart. In contrast to dityrosine, perfusate nitrotyrosine was not statistically significantly increased in our present study. This can be explained by recent results showing that at relatively low level of $ONOO^-$, nitrotyrosine formation is suppressed in favor of dityrosine [24]. The source of $ONOO^-$ release in the heart was not determined in the present study. Coronary and endocardial endothelial cells, specific cardiac nerves, and cardiac myocytes may all potentially contribute to $ONOO^-$ formation in the heart, since all of these cells are able to synthesize NO and superoxide. We have also found that hyperlipidemia increases the plasma nitrotyrosine level, a marker for systemic $ONOO^-$ generation. This is in accordance with our previous study showing an increase in serum nitrotyrosine in rabbits with high-cholesterol diet [13]. The reason why nitrotyrosine level was increased in the plasma but not in the coronary effluent is not clear. However, an increased systemic $ONOO^-$ formation has a greater chance to increase nitrotyrosine level in the circulating plasma in vivo, whereas cardiac $ONOO^-$ formation has less chance to increase nitrotyrosine significantly in the perfusate as the perfusion buffer passes through the coronary circulation only once in the Langendorff preparation. It should be noted that dityrosine and nitrotyrosine have been criticised as being specific for $ONOO^-$, e.g. myeloperoxidase activity in the presence of nitrite may also lead to nitrotyrosine formation [24,25]. However, both myeloperoxidase activity and nitrite concentration are very low in granulocyte-free, Krebs-perfused hearts. This suggests that the myeloperoxidase pathway does not substantially contribute

to nitrotyrosine formation in our present study. Furthermore, biochemical data suggesting that $ONOO^-$ does not cause tyrosine nitration [26] have been recently refuted [27]. Nevertheless, $NO_2^{\cdot}$ radical may also contribute to nitrotyrosine formation [24].

The cytotoxic effects of $ONOO^-$ include lipid peroxidation, nitration of tyrosine residues, oxidation of sulfhydryl groups, DNA-strand breakage [28], and inhibition of mitochondrial respiration [20], leading to tissue injury, which manifests itself, e.g., as a depression in myocardial contractile function [29]. Many studies show that enhanced formation of $ONOO^-$ in the myocardium is cytotoxic to the heart and contributes to ischemia/reperfusion injury both in vitro and in vivo, the spontaneous loss of cardiac function, as well as cytokine-induced myocardial contractile failure in isolated rat hearts and in dogs in vivo [30–32]. Many studies show a correlation between $ONOO^-$ formation and deterioration of cardiac function [31]. Therefore, here we tested if increased $ONOO^-$ in hearts of cholesterol-fed rats leads to a deterioration of cardiac function. We have found a significant increase in LVEDP in the cholesterol-fed group. LVEDP elevation is the most sensitive parameter of cardiac dysfunction showing that the capability of the heart to relax is deteriorated. This finding is in accordance with a study by Schwemmer et al. who reported a substantial decline in myocardial contractile and relaxation parameters in hypercholesterolemic guinea-pig hearts [33].

To further test if an increase in LVEDP was due to $ONOO^-$ formation, we examined the effect of FeTPPS, a $ONOO^-$ decomposition catalyst, on cardiac performance in cholesterol-fed and control groups. FeTPPS catalyzes the isomerization of $ONOO^-$ to nitrate anion and thereby decreases its decomposition to highly reactive intermediates such as nitrogen dioxide and hydroxyl radical [34]. Our results show that pretreatment with FeTPPS normalizes LVEDP in the cholesterol-fed group, but it does not change LVEDP in the control group. This finding further suggests that hyperlipidemia induces $ONOO^-$ formation in the rat heart which leads to an increase in LVEDP. As the biochemical measurements were not repeated in the FeTPPS groups, some unspecific effects of FeTPPS on cardiac NO and superoxide formation may also account for the effect of FeTPPS, which is a limitation of this study.

Our present results do not clarify the exact cellular mechanisms by which cholesterol diet leads to an increased formation of superoxide and therefore $ONOO^-$. We used an isolated, crystalloid-perfused rat heart model in our present study. In this model, the direct effect of plasma lipids and the effect of atherosclerosis can be excluded, since Wistar rats show moderate increase in serum cholesterol level and no substantial functional atherosclerosis develops due to cholesterol diet [4,35,36]. Therefore, enhancement of $ONOO^-$ is most likely due to the accumulation of tissue/membrane cholesterol [2] rather than the direct acute effects of high serum lipids itself. However, we have also found that plasma MDA level, a marker for lipid peroxidation, was significantly increased in the cholesterol-fed group. Although the source of plasma MDA was not determined, one may speculate the role of oxidized lipoproteins. Recent evidence shows that oxidized low density lipoprotein (oxLDL) is deposited in the myocardium which leads to expression of oxLDL receptor (LOX-1) and thereby induces apoptosis and cardiac dysfunction [37,38].

In summary, hyperlipidemia stimulates $ONOO^-$ generation in the heart which leads to myocardial dysfunction. Targeting $ONOO^-$ with pharmacological tools may be an exciting new strategy to protect the heart and the vasculature in hyperlipidemia.

Acknowledgements

This work was supported by grants from the Hungarian Scientific Research Found (OTKA T029843), Hungarian Ministry of Education (FKFP-0340/2000 and FKFP-0057/2001) and Ministry of Health (ETT 51/2000), and the Hungarian National R&D Program (NFKP-1/040).

References

[1] Roberts WC. Preventing and arresting coronary atherosclerosis. Am Heart J 1995;130:580–600.
[2] Hexeberg S, Willumsen N, Rotevatn S, Hexeberg E, Berge RK. Cholesterol induced lipid accumulation in myocardial cells of rats. Cardiovasc Res 1993;27:442–446.
[3] Haukur M, Leeson TS. Comparative electron microscope studies of the myocardium in the adult rats fed normal and cholesterol diet. J Mol Cell Cardiol 1975;7:195–202.
[4] Ferdinandy P, Szilvassy Z, Horvath LI et al. Loss of pacing-induced preconditioning in rat hearts: role of nitric oxide and cholesterol-enriched diet. J Mol Cell Cardiol 1997;29:3321–3333.
[5] Szilvassy Z, Ferdinandy P, Szilvassy J et al. The loss of pacing-induced preconditioning in atherosclerotic rabbits: role of hypercholesterolaemia. J Mol Cell Cardiol 1995;27:2559–2569.
[6] Ferdinandy P, Szilvassy Z, Baxter GF. Adaptation to myocardial stress in disease states: is preconditioning a healthy heart phenomenon? Trends Pharmacol Sci 1998;19:223–229.
[7] Lefer AM, Ma XL. Decreased basal nitric oxide release in hypercholesterolemia increases neutrophil adherence to rabbit coronary artery endothelium. Arterioscler Thromb 1993;13:771–776.
[8] Simonet S, Porro de Bailliencourt J, Descombes JJ, Mennecier P, Laubie M, Verbeuren TJ. Hypoxia causes an abnormal contractile response in the atherosclerotic rabbit aorta. Implication of reduced nitric oxide and cGMP production. Circ Res 1993;72:616–630.
[9] Deliconstantinos G, Villiotou V, Stavrides JC. Modulation of particulate nitric oxide synthase activity and peroxynitrite synthesis in cholesterol enriched endothelial cell membranes. Biochem Pharmacol 1995;49:1589–1600.
[10] Balligand JL, Cannon PJ. Nitric oxide synthases and cardiac muscle. Autocrine and paracrine influences. Arterioscler Thromb Vasc Biol 1997;17:1846–1858.
[11] Dusting GJ. Nitric oxide in cardiovascular disorders. J Vasc Res 1995;32:143–161.

[12] White CR, Brock TA, Chang LY et al. Superoxide and peroxynitrite in atherosclerosis. Proc Natl Acad Sci USA 1994;91:1044–1048.
[13] Szilvassy Z, Csont T, Pali T, Droy-Lefaix MT, Ferdinandy P. Nitric oxide, peroxynitrite and cGMP in atherosclerosis-induced hypertension in rabbits: beneficial effects of cicletanine. J Vasc Res 2001;38:39–46.
[14] Csont T, Balogh G, Csonka C et al. Hyperlipidemia induced by high cholesterol diet inhibits heat shock response in rat hearts. Biochem Biophys Res Commun 2002;290:1535–1538.
[15] Ferdinandy P, Danial H, Ambrus I, Rothery RA, Schulz R. Peroxynitrite is a major contributor to cytokine-induced myocardial contractile failure. Circ Res 2000;87:241–247.
[16] Csonka C, Szilvassy Z, Fulop F et al. Classic preconditioning decreases the harmful accumulation of nitric oxide during ischemia and reperfusion in rat hearts. Circulation 1999;100:2260–2266.
[17] Schulz R, Nava E, Moncada S. Induction and potential biological relevance of a Ca^{2+}-independent nitric oxide synthase in the myocardium. Br J Pharmacol 1992;105:575–580.
[18] Beckman JS, Parks DA, Pearson JD, Marshall PA, Freeman BA. A sensitive fluorometric assay for measuring xanthine dehydrogenase and oxidase in tissues. Free Radic Biol Med 1989;6:607–615.
[19] Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol 1978;52:302–310.
[20] Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol 1996;271:C1424–C1437.
[21] White CR, Darley-Usmar V, Berrington WR et al. Circulating plasma xanthine oxidase contributes to vascular dysfunction in hypercholesterolemic rabbits. Proc Natl Acad Sci USA 1996;93:8745–8749.
[22] Miller Jr. FJ, Gutterman DD, Rios CD, Heistad DD, Davidson BL. Superoxide production in vascular smooth muscle contributes to oxidative stress and impaired relaxation in atherosclerosis. Circ Res 1998;82:1298–1305.
[23] Hille R, Nishino T. Flavoprotein structure and mechanism. 4. Xanthine oxidase and xanthine dehydrogenase. FASEB J 1995;9:995–1003.
[24] Hurst JK. Whence nitrotyrosine? J Clin Invest 2002;109:1287–1289.
[25] Eiserich JP, Hristova M, Cross CE et al. Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. Nature 1998;391:393–397.
[26] Pfeiffer S, Mayer B. Lack of tyrosine nitration by peroxynitrite generated at physiological pH. J Biol Chem 1998;273:27280–27285.
[27] Goldstein S, Czapski G, Lind J, Merenyi G. Tyrosine nitration by simultaneous generation of $NO^{\cdot}$ and $O_2^{\cdot-}$ under physiological conditions. How the radicals do the job. J Biol Chem 2000;275:3031–3036.
[28] Rubbo H, Darley-Usmar V, Freeman BA. Nitric oxide regulation of tissue free radical injury. Chem Res Toxicol 1996;9:809–820.
[29] Schulz R, Dodge KL, Lopaschuk GD, Clanachan AS. Peroxynitrite impairs cardiac contractile function by decreasing cardiac efficiency. Am J Physiol 1997;272:H1212–H1219.
[30] Yasmin W, Strynadka KD, Schulz R. Generation of peroxynitrite contributes to ischemia-reperfusion injury in isolated rat hearts. Cardiovasc Res 1997;33:422–432.
[31] Ferdinandy P, Schulz R. Peroxynitrite: toxic or protective in the heart? Circ Res 2001;88:E12–E13.
[32] Oyama J, Shimokawa H, Momii H et al. Role of nitric oxide and peroxynitrite in the cytokine-induced sustained myocardial dysfunction in dogs in vivo. J Clin Invest 1998;101:2207–2214.
[33] Schwemmer M, Sommer O, Bassenge E. Blockade of angiotensin signaling improves myocardial function in hypercholesterolemia independent of changes in eicosanoid release. Cardiovasc Drugs Ther 2000;14:317–327.
[34] Misko TP, Highkin MK, Veenhuizen AW et al. Characterization of the cytoprotective action of peroxynitrite decomposition catalysts. J Biol Chem 1998;273:15646–15653.
[35] Roach PD, Balasubramaniam S, Hirata F et al. The low-density lipoprotein receptor and cholesterol synthesis are affected differently by dietary cholesterol in the rat. Biochim Biophys Acta 1993;1170:165–172.
[36] Horton JD, Cuthbert JA, Spady DK. Regulation of hepatic 7α-hydroxylase expression and response to dietary cholesterol in the rat and hamster. J Biol Chem 1995;270:5381–5387.
[37] Ehara S, Ueda M, Naruko T et al. Elevated levels of oxidized low density lipoprotein show a positive relationship with the severity of acute coronary syndromes. Circulation 2001;103:1955–1960.
[38] Li D, Williams V, Liu L, Chen H. Expression of LOX-1 during ischemia-reperfusion and its role in determination of apoptosis and left ventricular dysfunction in rats. Circulation 2001;104:II-11.

IV

Basic Res Cardiol 98: 304 – 310 (2003)
DOI 10.1007/s00395-003-0412-0

ORIGINAL CONTRIBUTION

Zoltán Giricz
Csaba Csonka
Annamária Ónody
Tamás Csont
Péter Ferdinandy

Role of cholesterol-enriched diet and the mevalonate pathway in cardiac nitric oxide synthesis

Received: 17 September 2002
Returned for 1. revision: 26 September 2002
1. Revision received: 22 January 2003
Returned for 2. revision: 10 February 2003
2. Revision received: 3 March 2003
Accepted: 12 March 2003
Published online: 22 April 2003

Z. Giricz · C. Csonka · A. Ónody · T. Csont
P. Ferdinandy, MD, PhD (✉)
Cardiovascular Research Group,
Department of Biochemistry
University of Szeged
Dóm tér 9
Szeged, H-6720, Hungary
Tel.: +36-62/545-755
Fax: +36-62/545-097
E-Mail: peter@bioch.szote.u-szeged.hu

Abstract Cardiac stress adaptation is deteriorated in hyperlipidemia possibly due to deterioration of nitric oxide (NO) metabolism. However, inhibition of HMG-CoA reductase, a key enzyme in the mevalonate pathway, was shown to increase the level of endothelial NO-synthase (eNOS) mRNA. Here we studied the effect of dietary and pharmacologic modulation of the mevalonate pathway on cardiac NO synthesis. Rats were fed 2% cholesterol-enriched or normal diet for 8 weeks. Normal and cholesterol-fed animals were treated with farnesol, a major metabolite of the mevalonate pathway (2.2 mg/kg *i.p.*) or with the HMG-CoA reductase inhibitor lovastatin (3 × 5 mg/kg *per os* for 3 days, n = 5–6 in each group). Cardiac NO content was significantly decreased in cholesterol-fed rats as assessed by electron spin resonance spectroscopy, however, other treatments did not influence cardiac NO content. Cardiac activity of Ca^{2+}-dependent NOS was unaffected by cholesterol-diet and by treatment with either farnesol or lovastatin, as assessed by ^{14}C-citrullin assay. Ca^{2+}-independent NOS activity was negligible in all groups. Cardiac eNOS protein content measured by Western blotting was also unchanged in all groups. We conclude that cholesterol-diet decreases cardiac NO content, however, cholesterol diet-induced inhibition of the mevalonate pathway does not account for the decreased NO level in the heart, and that the mevalonate pathway does not influence cardiac NO biosynthesis.

Key words Hyperlipidemia – heart – nitric oxide – mevalonate pathway – superoxide

Introduction

High cholesterol level is regarded as an important factor in the development of ischemic heart diseases. The heart of hyperlipidemic/atherosclerotic patients adapts poorly to oxidative or other kinds of stress, showing that endogenous adaptive mechanisms against myocardial stress are impaired (30). In hypercholesterolemia, the mevalonate pathway might be downregulated due to cholesterol-induced inhibition of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, the rate-limiting enzyme of this pathway (30). Therefore, excess exogenous cholesterol inhibits formation of polyprenyl derivates (2, 16). Farnesol and geranylgeraniol, the most important polyprenyl products of the mevalonate pathway, are utilized for protein prenylation and synthesis of other polyprenyl derivatives such as ubiquinone. Prenylation of several proteins including nuclear protein lamin B and a number of GTP-binding regulatory proteins (G proteins) is a prerequisite for their physiological function (15). The highly lipophyl 15-carbon farnesyl, or the 20-carbon geranylgeranyl side chains of G proteins can anchor them into lipid membranes, which is essential

for their function in signal transduction pathways. Ubiquinone plays a major role in the mitochondrial electron-transport system and serves as an endogenous antioxidant, therefore, it protects the ischemic-reperfused myocardium in rats (17). Consequently, inhibition of polyprenyl biosynthesis may impair signal-transduction processes and the endogenous antioxidant defence system in the myocardium. Indeed, we have previously shown that ischemic adaptation of the myocardium was impaired in hypercholesterolemic rat hearts (12, 33), however, it was recaptured by treatment with farnesol showing that hyperlipidemia led to the inhibition of the mevalonate pathway (8).

In normal hearts, nitric oxide (NO) is synthesized by Ca^{2+}-dependent NO synthases (NOS) in cardiac myocytes, vascular, and endocardial endothelium and specific cardiac neurons. NO plays an important role in the regulation of coronary circulation, contractile function, and ischemic adaptation of the heart (22). Increasing number of evidence accumulated in recent years showing that high-cholesterol diet impairs NO – cyclic guanosine 3':5'-monophosphate (cGMP) signalling in both endothelial and nonendothelial cells (6, 12, 26). We have previously shown that cardiac NO level is significantly decreased in rats with experimental hyperlipidemia (12). In contrast to the aforementioned findings, inhibition of HMG-CoA reductase by statins has been shown to increase mRNA level of endothelial type NO-synthase (eNOS) in human endothelial cells (24, 25). It is not known, however, if inhibition of HMG-CoA reductase affects NO synthesis significantly in the heart tissue, and if the mevalonate pathway plays a role in the regulation of cardiac NO level.

Therefore, the aim of the present study was to investigate the effect of the modulation of the mevalonate pathway by dietary cholesterol and by the HMG-CoA reductase inhibitor lovastatin on cardiac NO level and NOS activities in rat hearts.

Here we show that although cholesterol diet-induced hyperlipidemia decreases cardiac NO level, it is not mediated by the mevalonate pathway, and that the mevalonate pathway does not play a significant role in cardiac NO synthesis.

Materials and methods

This investigation conforms the Guide for the care and use of laboratory animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and it was approved by local ethics committee.

Experimental protocol

Eighteen weeks old male Wistar rats were fed 2% cholesterol-enriched or normal diet for 8 weeks. The body weights of the animals after the diet period were 420 – 500 g, and there were no significant difference between normal and cholesterol-fed groups. Wistar rats were chosen for the study, since this species shows moderate increase in serum cholesterol level due to high cholesterol diet, and no substantial atherosclerosis develops, however, accumulation of tissue cholesterol leads to strong biochemical effects (19, 29). Therefore, any non-specific effect of coronary atherosclerosis resulting from decreased coronary perfusion can be excluded in this model (12). The 8-week cholesterol-enriched diet moderately increased serum cholesterol from 1.53 ± 0.08 to 1.96 ± 0.07 ($n = 6$, $p < 0.05$) and markedly increased serum triglyceride from 0.47 ± 0.04 mmol/L to 1.62 ± 0.07 ($n = 6$, $p < 0.05$) mmol/L, respectively, similarly to our previous studies (4, 8, 12).

Animals on normal or cholesterol-rich diet were treated either with the HMG-CoA reductase inhibitor lovastatin (3×5 mg/kg *per os* for 3 days at the end of diet), or farnesol, a major metabolite of the mevalonate pathway (2×2.2 mg/kg *i.p.*, 6 and 1 hours before isolation of hearts, $n = 5-6$ in each group).

Rats were then anesthetized with diethyl ether, heparin (500 U/kg *i.v.*) was given, and the heart was isolated and perfused for 1 min at 37 °C in Langendorff mode with Krebs-Henseleit buffer as described (3, 11) to wash out blood. Ventricular tissue was frozen for further measurements of NOS activities, and NOS protein.

In separate experiments, hearts were perfused in a "working" mode (3) to measure cardiac haemodynamic parameters such as heart rate (HR), coronary flow (CF), aortic flow (AF), and left ventricular end-diastolic pressure (LVEDP). HR was derived from the left ventricular pressure curve, CF was measured by collecting effluents from the right atrium in a measuring cylinder for a time period, and AF was measured by a calibrated rotameter (KDG Mobrey, Sussex, UK). LVEDP was measured by a pressure transducer (B. Braun, Melsungen, Germany) connected to a small polyethylene catheter which was inserted into the left ventricle.

Measurement of cardiac NO by electron spin resonance spectroscopy

To measure NO level in cardiac tissue directly, *in vivo* spin-trapping of NO was applied, followed by ESR analysis of left ventricular tissue samples as described (3, 5, 9, 39). The spin trap diethyl-dithio-carbamate (DETC, 200 mg/kg), 50 mg/kg $FeSO_4$ and 200 mg/kg sodium citrate were slowly administered intravenously into the femoral vein under ether anaesthesia. DETC dissolved in distilled

Basic Research in Cardiology, Vol. 98, No. 5 (2003)

water was injected separately from $FeSO_4$ and sodium citrate in 0.5 mL total volume to avoid precipitation of $Fe^{2+}(DETC)_2$. $FeSO_4$ and sodium citrate were dissolved in distilled water, the pH was set to 7.4 with 1 mol/L NaOH, and brought to 1 mL volume before injection. Five minutes after DETC, $FeSO_4$, and citrate treatment, hearts were isolated and perfused in the Langendorff mode for 1 minute to eliminate blood, and approximately 150 mg tissue samples of the left ventricles were placed into quartz ESR tubes, frozen in liquid nitrogen until assayed for ESR spectra of the NO-$Fe^{2+}(DETC)_2$ complex. $Fe^{2+}(DETC)_2$ has high affinity for NO while forming NO-$Fe^{2+}(DETC)_2$. The specific triplet signal of the NO-$Fe^{2+}(DETC)_2$ is superimposed on the dominant background spectra of $Cu^{2+}(DETC)_2$. The detection limit of NO by this ESR method is 0.05 nmol (27). ESR spectra was recorded with a Bruker ECS106 (Rheinstetten, Germany) spectrometer operating at X band with 100 kHz modulation frequency at a temperature of 160 K, using 10 mW microwave power to avoid saturation. Scans were traced with 2.85 G modulation amplitude, 340 G sweep width, and 3356 G central field as described (27). After subtraction of the background signal of $Cu^{2+}(DETC)_2$, analysis of NO content was performed with double integration. The values were expressed in arbitrary units.

Measurement of NOS activities

NOS activities in cardiac homogenate of frozen ventricular tissue were determined from the rate of conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline as described (9, 10). Samples were incubated for 25 min at 37 °C in the presence or absence of either EGTA (1 mmol/L) or EGTA plus N^G-monomethyl-L-arginine (1 mmol/L) to determine the level of Ca^{2+}-dependent and -independent NOS activities, respectively. NOS activities were expressed in pmol/min/mg protein. Protein concentrations of ventricular homogenates were determined by a bicinchoninic acid protein assay kit (Sigma St. Louis, MO).

Measurement of eNOS protein content (Western immunoblotting)

Approximately 200 mg frozen left ventricular samples were homogenized in a buffer containing 10 mmol/L HEPES, 0.32 mmol/L sucrose, 0.1 mmol/L EDTA, 1.0 mmol/L DTT, 10 µg/mL trypsin inhibitor, 10 µg/mL leupeptin, 2.1 µg/mL aprotinin, and 10 µL PMSF (pH 7.2), and centrifuged at 1.000 g for 10 min at 4 °C. Homogenates were further diluted in this buffer to allow loading of 25 µg of total protein in each lane of 8% polyacrylamide gel. Electrophoresis was conducted at 200 V, 8 mA for 1.5 h, and proteins were transferred onto nitrocellulose membrane (25 V, 200 mA, 4 °C, 1.5 h) by Western blotting. Nitrocellulose sheets were then washed overnight in TBS-Tween-20 (0.2%, v/v) containing 5% dried skimmed milk powder to block nonspecific binding sites. After washing, membranes were incubated at room temperature for 1 h with monoclonal anti-eNOS antibody (Transduction Laboratories Lexington, KY) at 1:200 dilution. After repeated washing in TBS with 0.2% Tween-20, membranes were incubated with horseradish peroxidase-conjugated polyclonal goat anti-mouse IgG (Transduction Laboratories Lexington, KY) at 1:500 dilution at room temperature for 2 h. Membranes were then washed and developed to perform chemiluminescence detection with enhanced chemiluminescence kit (NEN Boston, MA) and exposed to X-ray film. Cardiac eNOS protein level was measured by scanning the immunoblots by sheet scanner. Band density was calculated by integrating the area (in pixels) and expressed in arbitrary units.

Measurement of superoxide production

To assess if hyperlipidemia leads to increased cardiac superoxide formation, in separate experiments, superoxide production in freshly minced ventricles was assessed by lucigenin-enhanced chemiluminescence in normal and hyperlipidemic control groups (9, 37). Approximately 100 mg of the apex of the heart was placed in 1 mL air-equilibrated Krebs-Henseleit solution containing 10 mmol/L HEPES-NaOH (pH 7.4) and 5 µmol/L lucigenin (9). Chemiluminescence was measured at room temperature in a liquid scintillation counter using a single active photomultiplier positioned in out-of-coincidence mode in the presence or absence of the superoxide scavenger nitro blue tetrazolium (NBT, 200 µmol/L). NBT-inhibitable chemiluminescence was considered an index of myocardial superoxide generation.

Statistics

Data were expressed as means ± SEM and analysed with one-way ANOVA followed by Bonferroni test. $P < 0.05$ was accepted as statistically significant difference.

Results

Cardiac function

Cholesterol-rich diet increased LVEDP, however, other cardiac functional parameters were not affected significantly when compared to controls. Additional farnesol

Table 1 Cardiac functional parameters in isolated rat hearts

Groups	HR	CF	AF	LVEDP
Control	269 ± 8	22.4 ± 0.5	44.3 ± 2.2	0.48 ± 0.05
Cholesterol-fed	265 ± 10	21.3 ± 0.6	45.7 ± 2.5	0.87 ± 0.06*
Farnesol	271 ± 8	22.9 ± 0.7	43.6 ± 2.3	0.50 ± 0.04
Chol+Far	265 ± 9	21.4 ± 0.4	44.9 ± 1.5	0.83 ± 0.06*
Lova	272 ± 7	22.0 ± 0.5	41.9 ± 2.1	0.51 ± 0.06
Chol+Lova	267 ± 9	21.7 ± 0.8	47.1 ± 1.9	0.90 ± 0.07*

Normal or cholesterol-fed animals were treated with farnesol (Farnesol; Chol+Far), or with lovastatin (Lova; Chol+Lova). Heart rate (HR, beats/min); Coronary flow (CF, mL/min); Aortic flow (AF, mL/min); Left ventricular end-diastolic pressure (LVEDP, kPa). Values are means ± SEM (n = 6 in each group). *P < 0.05 significant difference vs. control.

or lovastatin treatments either in the normal or in the cholesterol-fed groups were without effect (Table 1).

Cardiac NO content

After an 8-week cholesterol-enriched diet, myocardial NO level was significantly decreased in cholesterol-fed animals when compared to normal rats as assessed by electron spin spectroscopy after *in vivo* spin trapping. To test if the decreased NO level in cholesterol-fed rats is due to inhibition of the mevalonate pathway, cholesterol-fed rats were treated with farnesol, a major metabolite of the mevalonate pathway. Farnesol treatment did not influence the attenuated NO level in the cholesterol-fed group or the basal NO level in the normal group. To further test if a direct inhibition of HMG-CoA reductase changes NO content, normal and cholesterol-fed rats were treated with lovastatin. Lovastatin failed to change cardiac NO when compared to nontreated normal or cholesterol-fed groups, respectively (Fig. 1).

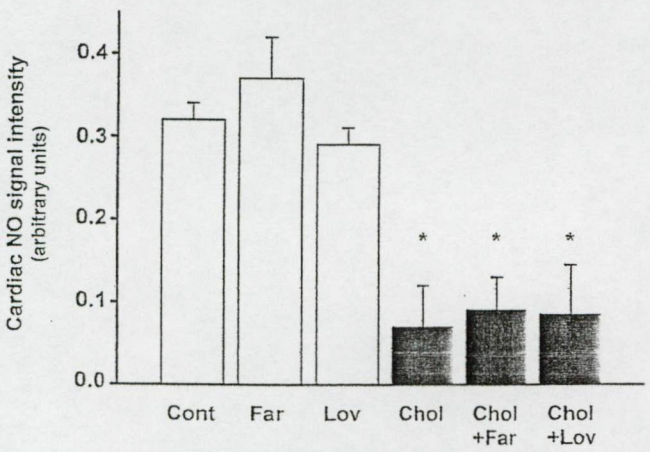


Fig. 1 Cardiac NO level measured by electron spin resonance spectroscopy in rat hearts. Rats were fed 2% cholesterol-enriched (Chol) or normal diet (Cont) for 8 weeks. Normal and cholesterol-fed animals were treated with farnesol (Far; Chol+Far), a major metabolite of the mevalonate pathway (2.2 mg/kg *i.p.*), or with the HMG-CoA reductase inhibitor lovastatin (Lov; Chol+Lov, 3 × 5 mg/kg *per os* for 3 days, n = 5–6 in each group). Data are mean ± SEM. *p < 0.05 vs. controls.

Cardiac NOS activities and eNOS protein levels

To test if changes in cardiac NO level is due to different rate of NO synthesis, NOS activities and NOS protein contents were assayed in all groups. Cardiac Ca^{2+}-dependent NOS activity was not influenced by cholesterol-enriched diet when compared to normal controls. Neither farnesol, nor lovastatin influenced cardiac NOS activities (Fig. 2A.). Similarly to NOS activities, cardiac eNOS protein content was not influenced by the different treatments (Fig. 2B.). As cardiac calcium-independent

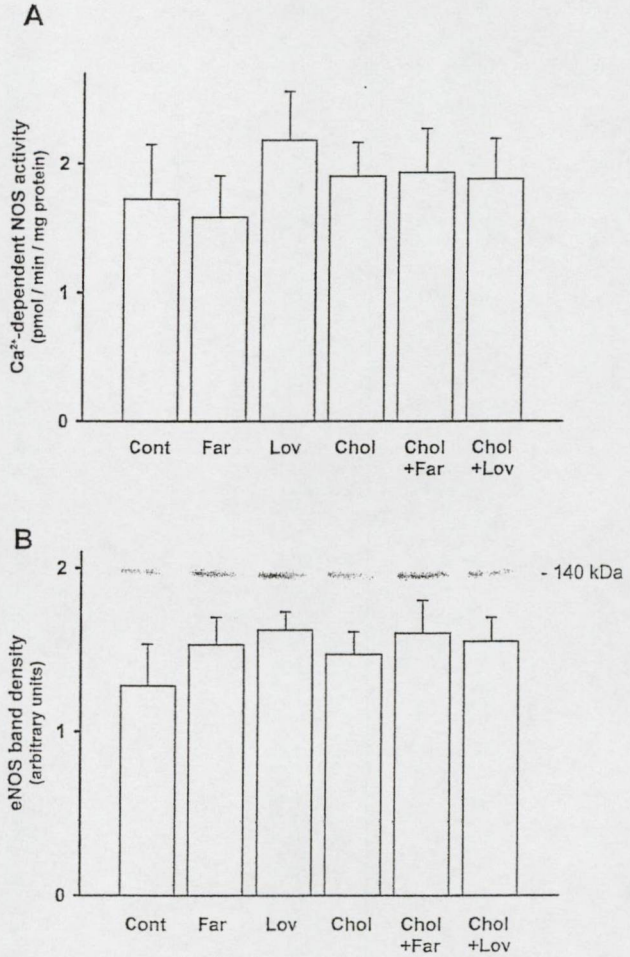


Fig. 2 Cardiac eNOS activity assessed by a ^{14}C-citrulline assay (A) and cardiac eNOS protein content measured by western blotting (B). Rats were fed 2% cholesterol-enriched (Chol) or normal diet (Cont) for 8 weeks. Normal and cholesterol-fed animals were treated with farnesol (Far; Chol+Far), a major metabolite of the mevalonate pathway (2.2 mg/kg *i.p.*), or with the HMG-CoA reductase inhibitor lovastatin (Lov; Col+Lov, 3 × 5 mg/kg *per os* for 3 days, n = 5–6 in each group). Data are mean ± SEM.

Basic Research in Cardiology, Vol. 98, No. 5 (2003)

(iNOS) activity was below the detection limit in all groups, cardiac iNOS protein content was not measured.

Cardiac superoxide production

To investigate if cardiac NO level is decreased in cholesterol-fed animals due to increased free radical formation, we measured superoxide-anion production in hearts of normal and cholesterol-fed animals by lucigenin-enhanced chemiluminescence assay. Cholesterol-enriched diet significantly increased superoxide production (control: 146.9 ± 9.4, hyperlipidemic: 366.4 ± 35.0 cpm × mg^{-1} wet weight, $p < 0.05$, $n = 6$ and 7).

Discussion

We have found that high cholesterol diet-induced decrease in cardiac NO level was not associated with changes in cardiac NOS activities and NOS protein content. The HMG-CoA reductase inhibitor lovastatin, or farnesol, a major metabolite of the mevalonate pathway, did not restore cardiac NO level in cholesterol-fed rats. Neither farnesol, nor lovastatin changed cardiac NO level, NOS activities, and NOS protein in normal rats. This is the first demonstration that cardiac NO synthesis is not affected significantly by pharmacologic or dietary modulation of the mevalonate pathway in rats. We have also found here that cardiac superoxide generation is enhanced in cholesterol-fed rats, which may explain the decreased cardiac NO level in hyperlipidemia.

A number of evidences suggest that HMG-CoA reductase inhibitors (statins) affect vascular NO production in different experimental models. Inhibition of HMG-CoA reductase by statins blocks hypoxia-mediated downregulation of eNOS and increases eNOS mRNA level in the mouse aorta (1, 24) and in hypercholesterolemic rabbits (21). Endres et al. (7) found that simvastatin increased the activity of calcium-dependent NOS in the mouse aorta by 2- to 3-fold. Increased NO level has been reported in cultured bovine aortic endothelial cells after simvastatin treatment (20). Although these findings suggest the role of the mevalonate pathway in the regulation of vascular NO production, the exact mechanisms remained unknown.

In contrast to the aforementioned findings in vascular tissue, we have found here that in the heart tissue, neither hyperlipidemia nor inhibition of HMG-CoA reductase by lovastatin affected NOS activities and NOS protein content. This may show that the regulation of NOS activities and NOS protein synthesis is not affected significantly by the mevalonate pathway in the heart tissue.

It is well known that high cholesterol level influences NO-cGMP signalling pathway. Lefer and Ma observed a reduced NO release from rabbit aorta in hypercholesterolemia (26). Deliconstantinos et al. showed that incorporation of high concentration of cholesterol into endothelial cell membranes caused a downregulation of NO synthesis in cultured bovine aortic endothelial cells (6). Feron et al. found that in cultured bovine aortic endothelial cells, experimental hypercholesterolemia decreased NO production but did not affect eNOS protein content (14). Further studies in this model showed that hypercholesterolemia-induced deterioration of NO production, measured in a single cell by NO selective electrode, was due to increased production of caveolin-1 (13). In contrast to these findings, increased eNOS level and NO production were found in cholesterol treated cultured bovine aortic endothelial cells (28). We have found in the present study that cholesterol diet-induced hyperlipidemia although decreases NO level, but it does not change NOS activity and NOS protein content in the rat heart. This shows that the decreased cardiac NO level is not due to impaired NO synthesis, however, it suggests that the breakdown of cardiac NO is increased due to hyperlipidemia. The mechanism of decreased cardiac NO level in hyperlipidemia remains unknown. However, it is well known that hyperlipidemia leads to increased production of reactive oxygen species (ROS) in the vasculature which leads to formation of peroxynitrite (18, 23, 31, 32, 34, 36). We have found here an increased superoxide production in hyperlipidemic hearts, so it is plausible to speculate that elevated ROS production is responsible for decreased NO level in hyperlipidemic myocardium. The source of increased superoxide formation in hyperlipidemic hearts has not been determined in the present study. It has been previously shown that hypercholesterolemia leads to increased angiotensin II levels and angiotensin receptor subtype 1 expression, which upregulates NADPH oxidase activity, a major source of superoxide in the vasculature (35, 38). Therefore, it is plausible to speculate that increased NADPH oxidase activity due to hyperlipidemia is a major source of increased superoxide production in hyperlipidemic hearts.

The major limitation of the present study is that no direct evidences were provided showing the magnitude of the blockade of the mevalonate pathway in the myocardium either by cholesterol diet or lovastatin treatment. However, the dose of lovastatin used in the present study is well above the human therapeutic range and it is comparable to other studies using lovastatin to investigate the role of the mevalonate pathway in NOS expression (1, 21, 24, 25). Nevertheless, it cannot be excluded that cholesterol diet and lovastatin treatment ineffectively blocked the mevalonate pathway.

Taken together, this is the first demonstration that modulation of the mevalonate pathway does not change the bioavailability of NO in heart. Furthermore, choles-

terol-enriched diet-induced decrease in NO bioavailability in the heart is not dependent on the mevalonate pathway. Therefore, to restore NO level in the heart in hyperlipidemia, pharmacological manipulation of the mevalonate pathway does not seem to be a valid option.

Acknowledgments This work was supported by grants from the Hungarian Scientific Research Found (OTKA T029843), Hungarian Ministry of Education (FKFP-0340/2000 and FKFP-0015/2001) and Ministry of Health (ETT 51/2000), and the Hungarian National R&D Program (NFKP-1/040).

References

1. Amin-Hanjani S, Stagliano NE, Yamada M, Huang PL, Liao JK, Moskowitz MA (2001) Mevastatin, an HMG-CoA reductase inhibitor, reduces stroke damage and upregulates endothelial nitric oxide synthase in mice. Stroke 32: 980–986
2. Casey PJ (1992) Biochemistry of protein prenylation. J Lipid Res 33: 1731–1740
3. Csonka C, Szilvassy Z, Fulop F, Pali T, Blasig IE, Tosaki A, Schulz R, Ferdinandy P (1999) Classic preconditioning decreases the harmful accumulation of nitric oxide during ischemia and reperfusion in rat hearts. Circulation 100: 2260–2266
4. Csont T, Balogh G, Csonka C, Boros I, Horvath I, Vigh L, Ferdinandy P (2002) Hyperlipidemia induced by high cholesterol diet inhibits heat shock response in rat hearts. Biochem Biophys Res Commun 290: 1535–1538
5. Csont T, Szilvassy Z, Fulop F, Nedeianu S, Pali T, Tosaki A, Dux L, Ferdinandy P (1999) Direct myocardial anti-ischaemic effect of GTN in both nitrate-tolerant and nontolerant rats: a cyclic GMP-independent activation of KATP. Br J Pharmacol 128: 1427–1434
6. Deliconstantinos G, Villiotou V, Stavrides JC (1995) Modulation of particulate nitric oxide synthase activity and peroxynitrite synthesis in cholesterol enriched endothelial cell membranes. Biochem Pharmacol 49: 1589–1600
7. Endres M, Laufs U, Huang Z, Nakamura T, Huang P, Moskowitz MA, Liao JK (1998) Stroke protection by 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors mediated by endothelial nitric oxide synthase. Proc Natl Acad Sci USA 95: 8880–8885
8. Ferdinandy P, Csonka C, Csont T, Szilvassy Z, Dux L (1998) Rapid pacing-induced preconditioning is recaptured by farnesol treatment in hearts of cholesterol-fed rats: role of polyprenyl derivatives and nitric oxide. Mol Cell Biochem 186: 27–34
9. Ferdinandy P, Danial H, Ambrus I, Rothery RA, Schulz R (2000) Peroxynitrite is a major contributor to cytokine-induced myocardial contractile failure. Circ Res 87: 241–247
10. Ferdinandy P, Panas D, Schulz R (1999) Peroxynitrite contributes to spontaneous loss of cardiac efficiency in isolated working rat hearts. Am J Physiol 276: H1861–H1867
11. Ferdinandy P, Szilvassy Z, Droy-Lefaix MT, Tarrade T, Koltai M (1995) KATP channel modulation in working rat hearts with coronary occlusion: effects of cromakalim, cicletanine, and glibenclamide. Cardiovasc Res 30: 781–787
12. Ferdinandy P, Szilvassy Z, Horvath LI, Csont T, Csonka C, Nagy E, Szentgyorgyi R, Nagy I, Koltai M, Dux L (1997) Loss of pacing-induced preconditioning in rat hearts: role of nitric oxide and cholesterol-enriched diet. J Mol Cell Cardiol 29: 3321–3333
13. Feron O, Dessy C, Desager JP, Balligand JL (2001) Hydroxy-methylglutaryl-coenzyme A reductase inhibition promotes endothelial nitric oxide synthase activation through a decrease in caveolin abundance. Circulation 103: 113–118
14. Feron O, Dessy C, Moniotte S, Desager JP, Balligand JL (1999) Hypercholesterolemia decreases nitric oxide production by promoting the interaction of caveolin and endothelial nitric oxide synthase. J Clin Invest 103: 897–905
15. Finegold AA, Schafer WR, Rine J, Whiteway M, Tamanoi F (1990) Common modifications of trimeric G proteins and ras protein: involvement of polyisoprenylation. Science 249: 165–169
16. Goldstein JL and Brown MS (1990) Regulation of the mevalonate pathway. Nature 343: 425–430
17. Hano O, Thompson-Gorman SL, Zweier JL, Lakatta EG (1994) Coenzyme Q10 enhances cardiac functional and metabolic recovery and reduces Ca^{2+} overload during postischemic reperfusion. Am J Physiol 266: H2174–H2181
18. Holland JA, Meyer JW, Schmitt ME, Sauro MD, Johnson DK, Abdul-Karim RW, Patel V, Ziegler LM, Schillinger KJ, Small RF, Lemanski LF (1997) Low-density lipoprotein stimulated peroxide production and endocytosis in cultured human endothelial cells: mechanisms of action. Endothelium 5: 191–207
19. Horton JD, Cuthbert JA, Spady DK (1995) Regulation of hepatic 7 alpha-hydroxylase expression and response to dietary cholesterol in the rat and hamster. J Biol Chem 270: 5381–5387
20. Kaesemeyer WH, Caldwell RB, Huang J, Caldwell RW (1999) Pravastatin sodium activates endothelial nitric oxide synthase independent of its cholesterol-lowering actions. J Am Coll Cardiol 33: 234–241
21. Kano H, Hayashi T, Sumi D, Esaki T, Asai Y, Thakur NK, Jayachandran M, Iguchi A (1999) A HMG-CoA reductase inhibitor improved regression of atherosclerosis in the rabbit aorta without affecting serum lipid levels: possible relevance of up-regulation of endothelial NO synthase mRNA. Biochem Biophys Res Commun 259: 414–419
22. Kinlay S, Libby P, Ganz P (2001) Endothelial function and coronary artery disease. Curr Opin Lipidol 12: 383–389
23. Kojda G, Harrison D (1999) Interactions between NO and reactive oxygen species: pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. Cardiovasc Res 43: 562–571
24. Laufs U, Fata VL, Liao JK (1997) Inhibition of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase blocks hypoxia-mediated down-regulation of endothelial nitric oxide synthase. J Biol Chem 272: 31725–31729
25. Laufs U, La F, V, Plutzky J, Liao JK (1998) Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. Circulation 97: 1129–1135

V

Defibrillatory Action of Glibenclamide Is Independent from ATP-Sensitive K^+ Channels and Free Radicals

Csaba Csonka, MD, PhD, Annamária Ónody, MSC, Tamás Csont, MD, PhD, and Péter Ferdinandy, MD, PhD

Abstract: This study investigated whether glibenclamide exerts a defibrillatory action and if this action is mediated by a blockade of ATP-sensitive K^+ channels (K_{ATP}) or by an anti-free radical mechanism. Aerobically perfused isolated rat hearts were subjected to 10 min of pacing-induced ventricular fibrillation (VF) followed by 10 min of perfusion without pacing (post-VF period), in the presence of solvent (controls), 1 μM K_{ATP} blocker glibenclamide, 10 μM K_{ATP} opener cromakalim, and their combination, respectively. In controls, pacing-induced VF caused a significant deterioration in cardiac function in the post-VF period. Spontaneous defibrillation was 42%. Glibenclamide improved post-VF cardiac function and resulted in 100% ($P < 0.05$) spontaneous defibrillation. Cromakalim did not significantly affect post-VF cardiac function and the incidence of spontaneous defibrillation as compared with controls. The combination of the compounds improved cardiac function and resulted in 83% ($P < 0.05$) spontaneous defibrillation. In separate experiments, 2,5-dihydroxybenzoic acid formation in the perfusate as a marker of hydroxyl radical formation was measured by high-performance liquid chromatography and cardiac superoxide production was assessed by lucigenin-enhanced chemiluminescence during pacing-induced VF. Glibenclamide did not affect hydroxyl radical generation or myocardial superoxide content during VF. The conclusion is that glibenclamide exerts a defibrillatory action and improves post-VF cardiac function in rat hearts and these effects are independent from K_{ATP} and free radicals.

Key Words: cromakalim, free radicals, glibenclamide, K_{ATP}, rat heart, ventricular fibrillation

(*J Cardiovasc Pharmacol*™ 2003;41: 916–922)

Cardiovascular Research Group, Department of Biochemistry, University of Szeged, Szeged, Hungary.

Received June 10, 2002; accepted January 23, 2003.

This work was supported by grants from the Hungarian Scientific Research Fund (OTKA T029843), Hungarian Ministry of Education (FKFP 0340/2000 and FKFP 0057/2001), Ministry of Health (ETT 51/2000), and the Hungarian National R&D Program (NFKP 1/040 and NFKP 1/001).

Address correspondence and reprint requests to Péter Ferdinandy, Cardiovascular Research Group, Department of Biochemistry, University of Szeged, Dóm tér 9, Szeged, Hungary. H-6720. E-mail: Peter@bioch.szote.u-szeged.hu. http://www.cardiovasc.com

Ventricular fibrillation (VF) is the most common cause of sudden cardiac death, which represents the single largest cause of "natural" death in the developed world.[1,2] Moreover, VF occurs commonly in patients after myocardial infarction as well as after cardiac surgery. The dominant therapeutic tools for patients with life-threatening ventricular arrhythmias have become the different defibrillators. Although defibrillators reduce cardiac mortality and anti-arrhythmic drugs alone generally are not thought to improve survival, concomitant therapy with anti-arrhythmic drugs in defibrillator recipients remains common. The use of anti-arrhythmic drug therapy in combination with implantable cardioverter defibrillator is synergistic in terms of beneficial effects but also has the potential for adverse interactions. Therefore, to examine the evidence for "hybrid" therapy, using combinations of drugs and nonpharmacologic treatments to reduce the incidence of VF and to increase spontaneous defibrillation are of great clinical importance. Two conditions appear to be important for the initiation of VF: abnormal myocardium, coronary arteries, or cell membrane ion channels; and transient modulating events, such as ischemia. Unfortunately, the exact mechanism of VF is still poorly understood.[3]

Glibenclamide is an oral, second-generation sulfonylurea drug, commonly used for treatment of non-insulin dependent diabetes mellitus. Glibenclamide blocks ATP-sensitive potassium channels (K_{ATP}) in the membrane of pancreatic ß-cells and leads to insulin secretion. K_{ATP} was first described by Noma[4] in cardiomyocytes. Two forms of K_{ATP} have been identified: a sarcolemmal form and another form in the mitochondrial inner membrane.[5,6] Glibenclamide has been widely used and well established as an effective, nonselective K_{ATP} blocker. Although opening of K_{ATP} has been shown to produce myocardial anti-ischemic effect[7] and to play a major role in ischemic preconditioning,[8,9] it is believed to favor the development of reentry arrhythmias including VF. K_{ATP} blockers protect ischemic myocardial cells against action potential shortening,[10] thereby exerting anti-arrhythmic effects and attenuating proarrhythmic effects of K_{ATP} openers. Therefore, K_{ATP} blockers, such as glibenclamide, show anti-arrhythmic including some anti-fibrillatory effects in several animal mod-

els of ischemia/reperfusion-induced arrhythmias[11–16] as well as in humans.[17] In an isolated rat heart model, glibenclamide has been found to exert a defibrillatory action after regional ischemia.[18] VF suppression produced by the combination of glibenclamide and the K_{ATP} opener RP 49356 raised the possibility that glibenclamide-induced defibrillation is mediated by a pharmacologic mechanism distinct from the modulation of K_{ATP}.[18] We have previously shown that pacing-induced VF results in deterioration of cardiac function after termination of VF and that this is mediated at least in part by VF-induced free radical formation.

Therefore, the objectives of this study were to investigate whether glibenclamide exerts a defibrillatory action and whether this action is mediated by a blockade of K_{ATP} in a pacing-induced VF model without ischemia/reperfusion in isolated rat hearts. We also tested whether glibenclamide-induced defibrillation is mediated by an inhibition of free radical formation, and whether glibenclamide is able to attenuate deterioration of cardiac mechanical function after VF.

Here we show that glibenclamide exerts a defibrillatory action in a pacing-induced VF model and improves post-VF cardiac function. These effects are not dependent on the blockade of K_{ATP} or inhibition of free radical formation.

METHODS

The investigation conforms to the *Guide for the care and use of laboratory animals* published by the US National Institutes of Health (publication no. 85–23, revised 1996) and was approved by local ethics committees.

Chemicals

Cromakalim (SmithKline Beecham, Essex, U.K.) and glibenclamide (Sigma, St. Louis, MO, U.S.A.) were dissolved in dimethylsulfoxide and the perfusion buffer contained the different compounds throughout the experimental protocols. The final concentration of dimethylsulfoxide was 5×10^{-3}% (vol/vol) in each group, which does not influence cardiac function.[19,20] All other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.).

Isolated Rat Heart Preparation

Male Wistar rats (300–350 g) were anesthetized with diethyl ether. After IV administration of heparin (500 U/kg), hearts were isolated and perfused in Langendorff mode or in a working mode.[21] Perfusion was carried out with an oxygenated Krebs-Henseleit buffer with the following components (in m*M*): 118.4 NaCl, 4.1 KCl, 2.5 $CaCl_2$, 25 $NaHCO_3$, 1.17 KH_2PO_4, 1.46 $MgCl_2$, and 11.1 glucose. Preload (1.7 kPa) and afterload (9.8 kPa) were kept constant throughout the experiments. The perfusion buffer was continuously equilibrated with 95% O_2 and 5% CO_2 (pH 7.4; 37°C). Heart rate, left ventricular developed pressure (LVDP), maximum value of the first derivative of LVDP ($+dP/dt_{max}$), and left ventricular end-diastolic pressure were measured from the left intraventricular pressure curve. Left ventricular pressure was measured by means of a pressure transducer (B. Braun, Melsungen, Germany) connected to a small polyethylene catheter inserted into the left ventricle through the left atrial cannula.[21,22] Ventricular pressure was on-line digitized and recorded. Coronary flow was measured by collecting effluent from the right atrium in a measuring cylinder for a timed period. Aortic flow was measured by a calibrated rotameter (KDG Flowmeters, Sussex, U.K.).[21]

Epicardial electrocardiogram was monitored throughout the experimental period by two silver electrodes attached directly to the surface of the heart to determine VF.

Induction of Ventricular Fibrillation by Electrical Pacing

VF was induced by rapid electrical pacing (20 Hz, 1,200 beats/min) for 10 min using (double-threshold) square wave impulses of 1-ms duration by an electric stimulator (Experimetria, Budapest, Hungary) through silver electrodes.[23] The active lead was attached directly to the surface of the apex of the heart and the ground lead was attached to the metal aortic cannula. These conditions suppressed the sinus node activity and resulted in VF. VF started immediately after commencement of rapid pacing. During the pacing-induced VF, the hearts were switched to Langendorff perfusion to maintain coronary perfusion.

Experimental Protocol

The experimental protocol is shown in Figure 1.

First Series of Experiments

In the first series of experiments we investigated the effect of glibenclamide on spontaneous defibrillation and post-VF cardiac function. The perfusion fluid contained solvent, 1 μ*M* K_{ATP} blocker glibenclamide, 10 μ*M* nonselective K_{ATP} opener cromakalim, and their combination throughout the experimental protocol, respectively. The concentration of glibenclamide and cromakalim was selected according to our previous studies showing that glibenclamide at 1 μ*M* was the most effective concentration to antagonize the maximal anti-ischemic effect of the K_{ATP} opener cromakalim at 10 μ*M* in rat hearts[19] and to block the protective effect of ischemic preconditioning.[24] Glibenclamide at higher doses was found to worsen basal and postischemic myocardial function, and the most effective anti-ischemic concentration of cromakalim was 10 μ*M* in isolated working rat hearts.[19] Rat hearts were perfused for 10 min in "working" perfusion to record baseline parameters for cardiac performance. Hearts (n = 12 in each group) were then subjected to 10 min VF induced by pacing at 20 Hz under Langendorff perfusion as described.[23] After termination of pacing, 5 min Langendorff perfusion was applied to allow spontaneous defibrillation before switching to work-

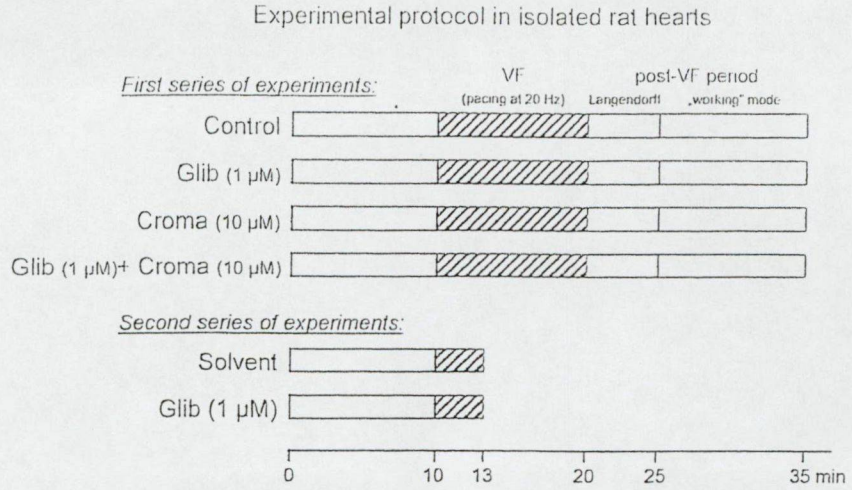


FIGURE 1. Isolated rat hearts (n = 12 in each group) were perfused for 10 min in "working" perfusion to record baseline parameters for cardiac performance, then subjected to 10 min ventricular fibrillation (VF) induced by pacing at 20 Hz under Langendorff perfusion. After termination of pacing, 5 min of Langendorff perfusion was applied to allow spontaneous defibrillation before switching to working perfusion (post-VF period, open bars). Incidence of spontaneous defibrillation was measured in the post-VF period. When VF was spontaneously terminated or after mechanical defibrillation when sinus rhythm was restored, the perfusion was converted to working mode for an additional 10 min to measure post-VF cardiac function. The perfusion fluid contained solvent (control), 1 μ*M* glibenclamide (Glib), 10 μ*M* cromakalim (Croma), and their combination (Glib + Croma) throughout the experiment, respectively. In separate experiments, hearts were subjected to 3 min pacing-induced VF and 2,5-dihydroxybenzoic acid formation and cardiac superoxide production were measured (n = 7 in each group).

ing perfusion. If VF was spontaneously terminated and sinus rhythm was restored, the perfusion was converted to working mode for an additional 10 min to measure post-VF cardiac function. In hearts not showing spontaneous defibrillation, mechanical defibrillation was attempted after the 5-min Langendorff perfusion period to measure post-VF cardiac function. In case of unsuccessful defibrillation, post-VF cardiac function was not measured.

Second Series of Experiments

In the second series of experiments we investigated the effect of glibenclamide on oxygen free radical formation in separate experiments. The perfusion buffer contained 1 μ*M* glibenclamide or its solvent throughout the experiments. After 10 min working perfusion, hearts (n = 7 in each group) were subjected to 3 min pacing-induced VF. Our previous study demonstrated a maximal oxygen free radical formation at the 3rd min of pacing-induced VF.[23] Coronary effluent was collected for 30 seconds at the 3rd min of pacing-induced VF and 2,5-dihydroxybenzoic acid (2,5-DHBA) content as a marker of hydroxyl radical formation was immediately measured by high-performance liquid chromatography (HPLC). In addition, approximately 150 mg cardiac tissue from the apex of the left ventricle was collected and cardiac superoxide production was measured by lucigenin-enhanced chemiluminescence in freshly minced tissue as described.[25]

Measurement of Hydroxyl Radical Formation

To measure cardiac formation of hydroxyl radicals in the second series of experiments, the perfusate was supplemented with 1 m*M* salicylate.[26] The formation of 2,5-DHBA, the product of the reaction of salicylate and hydroxyl radical,[27] was measured from the coronary effluent at the 3rd min of pacing-induced VF. Coronary effluent was filtered through a Millex-HV filter (0.45 μm pore size, Nihon Millipore, Ltd., Yonezawa, Japan). Each sample (20 μl) was injected onto a Merck 50734 LiChrospher 100 RP-18 cartridge (Merck, Darmstadt, Germany). The HPLC unit consisted of a Gilson 307 pump, an ESA (Chelmsford, MA, U.S.A.) Model 5011 analytical cell, and an ESA Coulochem II electrochemical detector. Elution buffer contained 50 m*M* sodium acetate, 50 m*M* citric acid, 25% methanol, and 5% i-propanol (pH 2.5). The flow rate was 0.5 ml/min. The detection potential was maintained at 750 mV. Peaks were identified by authentic standard (2,5-dihydroxybenzoic acid, Sigma, St. Louis, MO, U.S.A.) as well as by injecting the hydroxylated products of salicylic acid from a pure hydroxyl radical generating system, i.e., Fenton reaction (50 μ*M* $FeSO_4$ and 100 μ*M* H_2O_2 final concentrations).

In separate experiments, we also tested whether glibenclamide directly scavenges hydroxyl radical in vitro. Fenton reaction was used to generate hydroxyl radicals in the presence and absence of 1 μ*M* and 10 μ*M* glibenclamide or its solvent dimethylsulfoxide (0.1, 1% [vol/vol]). Formation of 2,5-DHBA was measured as described previously.

Measurement of Superoxide Radical, a Marker for Reactive Oxygen Species Formation

In the second series of experiments, myocardial superoxide production in freshly minced cardiac tissue from the apex of the left ventricle was assessed by lucigenin-enhanced

chemiluminescence method using 5 μ*M* lucigenin as described.[25] Chemiluminescence was measured in the presence and absence of the superoxide scavenger nitro blue tetrazolium (200 μ*M*) using a scintillation counter (Packard, Meriden, CT, U.S.A.). Nitro blue tetrazolium–inhibitable chemiluminescence was considered an index of myocardial superoxide generation.

Statistics

Data were expressed as means ± SEM. Groups were analyzed with one-way analysis of variance followed by the Bonferroni test. Incidence of VF was compared by the Fisher Exact test between groups. $P < 0.05$ was accepted as indicating a statistically significant difference.

RESULTS

Cardiac Function and Spontaneous Defibrillation

In the solvent-treated control group, pacing-induced VF markedly decreased cardiac function in the post-VF period when compared with basal values (Fig. 2). After termination of pacing, the incidence of spontaneous defibrillation was 42% (Fig. 3).

The nonselective K_{ATP} blocker glibenclamide (1 μ*M*) did not influence basal cardiac hemodynamic parameters except coronary flow (Table 3). However, glibenclamide significantly improved cardiac function after pacing-induced VF (Fig. 2) and resulted in 100% ($P < 0.05$) spontaneous defibrillation (Fig. 3).

To investigate whether K_{ATP} plays a role in the mechanism of the defibrillatory effect and the improvement of post-VF cardiac function by glibenclamide, hearts were perfused in the presence of the combination of 1 μ*M* glibenclamide and 10 μ*M* cromakalim (a nonselective K_{ATP} opener) and 10 μ*M* cromakalim alone, respectively. Cromakalim alone did not affect post-VF cardiac function (Fig. 2) and the incidence of spontaneous defibrillation (42% NS, Fig. 3) as compared with controls. Cromakalim did not affect the effects of glibenclamide as the combination of the compounds significantly improved cardiac function and resulted in 83% spontaneous defibrillation (Fig. 2), effects similar to that of glibenclamide alone.

Cardiac Hydroxyl Radical Generation

To investigate whether the possible effects of glibenclamide are mediated by an inhibition of free radical formation, hearts were perfused in the presence of 1 m*M* salicylate, and cardiac 2,5-DHBA formation was measured from the coronary effluent by HPLC as a marker of cardiac hydroxyl radical production. Glibenclamide (1 μ*M*) did not affect cardiac hydroxyl radical generation at the 3rd min of pacing-induced VF when compared with nontreated controls (Table 1).

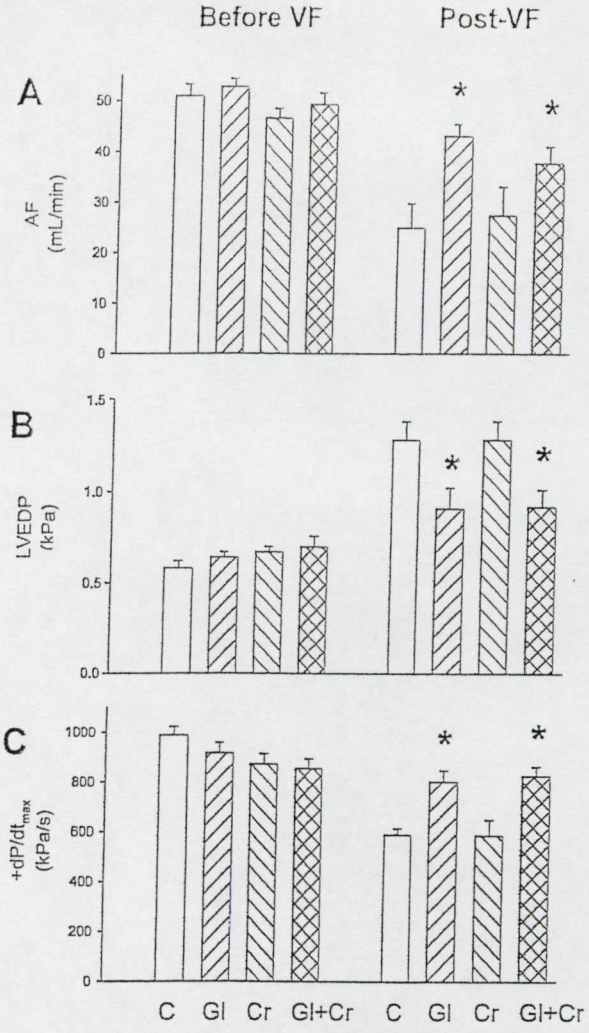


FIGURE 2. Effects of glibenclamide (Gl), cromakalim (Cr), their combination (Gl + Cr), and their solvent (control, C) on aortic flow (AF (A), left ventricular end-diastolic pressure (LVEDP) (B), and maximum value of the first derivative of LVDP ($+dP/dt_{max}$) (C) measured before pacing-induced ventricular fibrillation (VF) (n = 12 in each group) and at the 15th min of post-VF period (after VF, n = 9–12). Data are mean ± SEM, *$P < 0.05$ vs controls.

Hydroxyl Radical Generation in Vitro

We also investigated the effects of different concentrations of glibenclamide on hydroxyl radical formation in vitro. Hydroxyl radicals were produced by Fenton reaction in the presence of different concentrations of glibenclamide and its solvent. Glibenclamide (1 μ*M* and 10 μ*M*) did not inhibit 2,5-DHBA production. The solvent dimethylsulfoxide (0.1 and 1% [vol/vol]) concentration-dependently decreased 2,5-DHBA formation in vitro (Table 2); however, lower concentrations had no effect (data not shown).

Cardiac Superoxide Anion Generation

In addition to cardiac hydroxyl radical formation, we investigated the effect of 1 μ*M* glibenclamide on cardiac super-

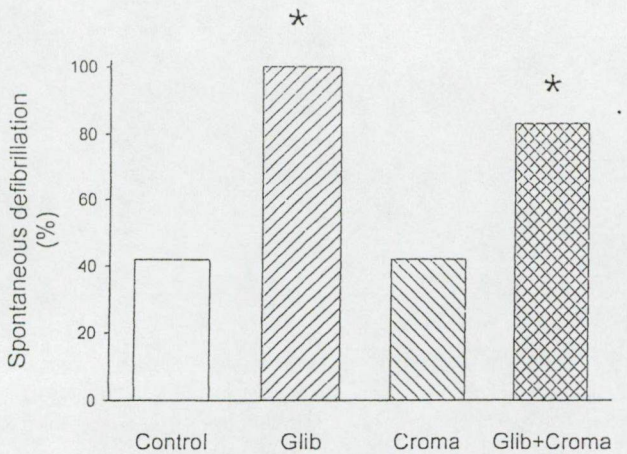


FIGURE 3. Incidence of spontaneous defibrillation after 10-min electrical pacing-induced ventricular fibrillation (VF) in the presence of glibenclamide (Glib), cromakalim (Croma), their combination (Glib + Croma), and their solvent (control), respectively. *$P < 0.05$ vs controls (n = 12 in each group).

oxide anion formation. Glibenclamide did not affect cardiac superoxide generation as assessed by lucigenin-enhanced chemiluminescence in freshly minced cardiac tissue when compared with solvent-treated controls at the 3rd min of pacing-induced VF (Table 1).

DISCUSSION

Our results show that the K_{ATP} blocker glibenclamide at a 1-μM concentration increases the incidence of spontaneous defibrillation and attenuates the deterioration of cardiac function after pacing-induced VF in isolated rat hearts. This is the first demonstration that glibenclamide exerts a potent defibrillatory effect and improves post-VF cardiac function in an electrical pacing-induced VF model in rat hearts. We further showed that the defibrillatory action of glibenclamide is independent from K_{ATP} blockade, as the nonselective K_{ATP} opener cromakalim did not modify the effect of glibenclamide. We also observed that these effects of glibenclamide are not dependent on any anti-oxidant mechanism.

K_{ATP} is known to play a dual role in cardiac arrhythmias. Activation of K_{ATP} may exert proarrhythmic effect via shortening of action potential. Tosaki et al.[28] suggested that exacerbations of reperfusion-induced arrhythmias by cromakalim are also associated with K^+ efflux and that the K_{ATP} blocker glibenclamide reduces the incidence of reperfusion-induced VF and ventricular tachycardia in isolated rat heart. D'Alonzo et al.[29] have also found that glibenclamide has potential anti-arrhythmic activity in isolated rat hearts. El-Reyani et al.[30] have shown that glibenclamide has no anti-arrhythmic effect but it improves survival after coronary artery ligation in rats by increasing the possibility of recovery from VF. Accordingly, glibenclamide has been found to induce spontaneous termination of VF after regional ischemia/reperfusion.[12] Rees and Curtis[18] reported that glibenclamide possesses defibrillatory activity after regional ischemia and raised the possibility that this effect of glibenclamide is independent from K_{ATP} blockade. Our present results showed that glibenclamide was able to induce spontaneous defibrillation after pacing-induced VF without ischemia and reperfusion and this action was not inhibited by the nonselective K_{ATP} opener cromakalim. Therefore, this is the first demonstration that glibenclamide exerts a defibrillatory action in the heart via a mechanism independent from K_{ATP} in a pacing-induced VF model.

We have previously shown that pacing-induced VF is capable of eliciting free radical formation even in the absence

TABLE 1. Effects of glibenclamide (Glib), cromakalim (Croma), their combination (Glib + Croma), and their solvent (Control) on heart rate (HR) and coronary flow (CF)

	Control	Glib (1 μM)	Croma (10 μM)	Glib (1 μM) + Croma (10 μM)
HR before	297 ± 7	260 ± 7	277 ± 7	288 ± 7
HR after	277 ± 6	258 ± 13	261 ± 7	271 ± 9
CF before	24.2 ± 1.1	21.5 ± 1.1*	27.3 ± 1.3	24.7 ± 1.1
CF after	26.1 ± 0.9	22.2 ± 2.5	26.3 ± 1.2	23.9 ± 0.8

Data are mean ± SEM. *$P < 0.05$ vs controls.
Values measured before pacing-induced VF (n = 12 in each group) and at the 15th min of post-VF period (after VF, n = 9–12 in each group).

TABLE 2. 2,5-dihydroxybenzoic acid (2,5-DHBA) formation measured by HPLC as a marker for cardiac OH radical generation and superoxide production

	Untreated	1 μM glibenclamide
2,5-DHBA formation (10^{-9} mol/min)	37.4 ± 9.20	31.8 ± 3.16
Lucigenin chemiluminescence (cpm/mg)	895 ± 126.5	896 ± 151.2

Data are means ± SEM.
Values measured by lucigenin-enhanced chemiluminescence in untreated control and glibenclamide treated hearts at the 3rd min of electrical pacing-induced VF (n = 7 in each group).

TABLE 3. Relative inhibition produced by glibenclamide (Glib) and dimethyl-sulfoxide (DMSO) on 2,5-dihydroxybenzoic acid (2,5-DHBA) formation as a marker of hydroxyl radical generation in vitro

2,5-DHBA formation	Glib (1 μM)	Glib (10 μM)
(% of untreated)	88.2 ± 20.2	82.0 ± 10.8
2,5-DHBA formation	0.1% DMSO	1% DMSO
(% of untreated)	20.0 ± 7.9*	3.9 ± 1.2*

Data are mean ± SEM. *$P < 0.05$ vs. untreated. n = 3 in each group.

of ischemia and reperfusion.[23] We have found here that the defibrillatory action of glibenclamide was not inhibited by the K_{ATP} opener cromakalim, which showed that this effect is K_{ATP}-independent. Therefore, we hypothesized that glibenclamide may exert its defibrillatory effect by scavenging oxygen free radicals. However, glibenclamide had no effect on either myocardial hydroxyl or superoxide radical formation in our present study. Moreover, glibenclamide did not influence hydroxyl radical formation in vitro as assessed by HPLC, showing that the defibrillatory action of glibenclamide may be independent from any antioxidant mechanism. This finding is in accordance with that of Noda et al.,[31] who found that glibenclamide did not scavenge superoxide and hydroxyl radicals in vitro as measured by electron spin resonance spectroscopy.

Opening of K_{ATP} leads to membrane hyperpolarization and shortening of action potential, both effects resulting in a reduction in intracellular calcium levels. Therefore, K_{ATP} openers are known as cardioprotective agents.[32] Moreover, recent findings suggest that opening of cardiac sarcolemmal[33] or mitochondrial[34,35] K_{ATP} increases myocardial free radical production, which could trigger cardioprotection.[32,34,35] However, it is well established that a pathologic increase in free radical production leads to a diminished cardiac performance and arrhythmias. Therefore, we can speculate that K_{ATP} blockers may indirectly decrease cardiac oxygen free radical production influenced by K_{ATP} activation, thereby preventing hearts against VF. Here we did not find changes in free radical production due to glibenclamide treatment; therefore, our present results do not support this hypothesis.

Unfortunately, the exact mechanism of glibenclamide-induced, K_{ATP}-independent defibrillation remains unknown. It has been shown that the effects of glibenclamide are not solely related to its action on K_{ATP}. Tominaga et al.[36] reported that glibenclamide inhibits chloride conductance in guinea pig ventricular myocytes, thereby exerting anti-arrhythmic effect. Glibenclamide has been shown to stimulate lactate production during normoxic perfusion[37] and to attenuate the increase in lactate production during hypoxia.[38] Rees and Curtis[18] showed that glibenclamide widened QT interval and caused sinus bradycardia during regional ischemia. This effect of glibenclamide was not antagonized by the K_{ATP} opener RP 49356. Picard et al.[39] showed that glibenclamide prevented the reperfusion-induced membrane arachidonic acid release. The aforementioned K_{ATP}-independent actions of glibenclamide may contribute to its defibrillatory effect.

It has been shown that reperfusion-induced VF significantly worsens cardiac mechanical function when measured after termination of VF and restoration of sinus rhythm and that this effect of VF is independent from the severity of ischemia.[40,41] We have further shown that electrically induced VF without ischemia and reperfusion also causes a deterioration in post-VF cardiac function.[23] The exact mechanism of VF-induced cardiac dysfunction is not completely known. Free radical formation,[23] Ca^{2+} overload,[42] reduced levels of ATP and creatine phosphate,[43] and alterations in mitochondrial gene expression[41] are thought to be responsible for reduced post-VF cardiac performance. We may speculate that VF-induced mild demand ischemia in the present pacing-induced VF model may account for the post-VF deterioration of cardiac function. However, glibenclamide is known to worsen the outcome of ischemia/reperfusion.[19] In our present study glibenclamide improved post-VF cardiac function, which argues against VF-induced demand ischemia as a cause of the deterioration of post-VF cardiac function. Nevertheless, this is the first demonstration that glibenclamide is able to attenuate VF-induced cardiac mechanical dysfunction; however, the mechanism of this effect remains unknown.

Taken together, we have found here that glibenclamide exerts a defibrillatory action and improves post-VF cardiac function in isolated rat heart and that these effects are independent from K_{ATP} and inhibition of free radical formation. The possible clinical application of these effects of glibenclamide in the treatment of sudden cardiac death in defibrillator recipient patients needs further investigation.

REFERENCES

1. Zheng ZJ, Croft JB, Giles WH, et al. Sudden cardiac death in the United States, 1989 to 1998. *Circulation*. 2001;104:2158–63.
2. Sotoodehnia N, Zivin A, Bardy GH, et al. Reducing mortality from sudden cardiac death in the community: lessons from epidemiology and clinical applications research. *Cardiovasc Res*. 2001;50:197–209.
3. Witkowski FX, Leon LJ, Penkoske PA, et al. Spatiotemporal evolution of ventricular fibrillation. *Nature*. 1998;392:78–82.
4. Noma A. ATP-regulated K+ channels in cardiac muscle. *Nature*. 1983; 305:147–8.
5. Inoue I, Nagase H, Kishi K, et al. ATP-sensitive K+ channel in the mitochondrial inner membrane. *Nature*. 1991;352:244–7.
6. Paucek P, Mironova G, Mahdi F, et al. Reconstitution and partial purification of the glibenclamide-sensitive, ATP-dependent K+ channel from rat liver and beef heart mitochondria. *J Biol Chem*. 1992;267:26062–9.
7. Grover GJ. Pharmacology of ATP-sensitive potassium channel (KATP) openers in models of myocardial ischemia and reperfusion. *Can J Physiol Pharmacol*. 1997;75:309–15.
8. Przyklenk K, Kloner RA. Ischemic preconditioning: exploring the paradox. *Prog Cardiovasc Dis*. 1998;40:517–47.
9. Gross GJ, Fryer RM. Sarcolemmal versus mitochondrial ATP-sensitive K+ channels and myocardial preconditioning. *Circ Res*. 1999;84:973–9.
10. Ferdinandy P, Csont T, Csonka C, et al. Capsaicin-sensitive local sensory innervation is involved in pacing-induced preconditioning in rat hearts: role of nitric oxide and CGRP? *Naunyn Schmiedebergs Arch Pharmacol*. 1997;356:356–63.
11. Gwilt M, Henderson CG, Orme J, et al. Effects of drugs on ventricular fibrillation and ischaemic K+ loss in a model of ischaemia in perfused guinea-pig hearts in vitro. *Eur J Pharmacol*. 1992;220:231–6.
12. Bril A, Laville MP, Gout B. Effects of glibenclamide on ventricular arrhythmias and cardiac function in ischaemia and reperfusion in isolated rat heart. *Cardiovasc Res*. 1992;26:1069–76.
13. Billman GE, Avendano CE, Halliwill JR, et al. The effects of the ATP-dependent potassium channel antagonist, glyburide, on coronary blood flow and susceptibility to ventricular fibrillation in unanesthetized dogs. *J Cardiovasc Pharmacol*. 1993;21:197–204.
14. Wilde AA. Role of ATP-sensitive K+ channel current in ischemic arrhythmias. *Cardiovasc Drugs Ther*. 1993;7(suppl 3):521–6.
15. Baczko I, Lepran I, Papp JG. KATP channel modulators increase survival rate during coronary occlusion-reperfusion in anaesthetized rats. *Eur J Pharmacol*. 1997;324:77–83.

16. Rees S, Curtis MJ. Which cardiac potassium channel subtype is the preferable target for suppression of ventricular arrhythmias? *Pharmacol Ther.* 1996;69:199–217.
17. Schotborgh CE, Wilde AA. Sulfonylurea derivatives in cardiovascular research and in cardiovascular patients. *Cardiovasc Res.* 1997;34:73–80.
18. Rees SA, Curtis MJ. Pharmacological analysis in rat of the role of the ATP-sensitive potassium channel as a potential target for antifibrillatory intervention in acute myocardial ischaemia. *J Cardiovasc Pharmacol.* 1995;26:280–8.
19. Ferdinandy P, Szilvassy Z, Droy-Lefaix MT, et al. KATP channel modulation in working rat hearts with coronary occlusion: effects of cromakalim, cicletanine, and glibenclamide. *Cardiovasc Res.* 1995;30:781–7.
20. Ferdinandy P, Koltai M, Tosaki A, et al. Cicletanine improves myocardial function deteriorated by ischemia/reperfusion in isolated working rat hearts. *J Cardiovasc Pharmacol.* 1992;19:181–9.
21. Csonka C, Szilvassy Z, Fulop F, et al. Classic preconditioning decreases the harmful accumulation of nitric oxide during ischemia and reperfusion in rat hearts. *Circulation.* 1999;100:2260–6.
22. Ferdinandy P, Szilvassy Z, Csont T, et al. Nitroglycerin-induced direct protection of the ischaemic myocardium in isolated working hearts of rats with vascular tolerance to nitroglycerin. *Br J Pharmacol.* 1995;115: 1129–31.
23. Ferdinandy P, Das DK, Tosaki A. Pacing-induced ventricular fibrillation leading to oxygen free radical formation in aerobically perfused rat hearts. *J Mol Cell Cardiol.* 1993;25:683–92.
24. Ferdinandy P, Szilvassy Z, Koltai M, et al. Ventricular overdrive pacing-induced preconditioning and no-flow ischemia-induced preconditioning in isolated working rat hearts. *J Cardiovasc Pharmacol.* 1995;25:97–104.
25. Ferdinandy P, Danial H, Ambrus I, et al. Peroxynitrite is a major contributor to cytokine-induced myocardial contractile failure. *Circ Res.* 2000;87: 241–7.
26. Tosaki A, Bagchi D, Pali T, et al. Comparisons of ESR and HPLC methods for the detection of OH radicals in ischemic/reperfused hearts: a relationship between the genesis of free radicals and reperfusion arrhythmias. *Biochem Pharmacol.* 1993;45:961–9.
27. Grootveld M, Halliwell B. Aromatic hydroxylation as a potential measure of hydroxyl-radical formation in vivo: identification of hydroxylated derivatives of salicylate in human body fluids. *Biochem J.* 1986;237: 499–504.
28. Tosaki A, Szerdahelyi P, Engelman RM, et al. Potassium channel openers and blockers: do they possess proarrhythmic or antiarrhythmic activity in ischemic and reperfused rat hearts? *J Pharmacol Exp Ther.* 1993;267: 1355–62.
29. D'Alonzo AJ, Darbenzio RB, Hess TA, et al. Effect of potassium on the action of the KATP modulators cromakalim, pinacidil, or glibenclamide on arrhythmias in isolated perfused rat heart subjected to regional ischaemia. *Cardiovasc Res.* 1994;28:881–7.
30. El-Reyani NE, Bozdogan O, Baczko I, et al. Comparison of the efficacy of glibenclamide and glimepiride in reperfusion-induced arrhythmias in rat. *Eur J Pharmacol.* 1999;365:187–192.
31. Noda Y, Mori A, Cossins E, et al. Gliclazide scavenges hydroxyl and superoxide radicals: an electron spin resonance study. *Metabolism.* 2000; 49:14–6.
32. Gross GJ. The role of mitochondrial KATP channels in cardioprotection. *Basic Res Cardiol.* 2000;95:280–4.
33. Obata T, Yamanaka Y. Block of cardiac ATP-sensitive K(+) channels reduces hydroxyl radicals in the rat myocardium. *Arch Biochem Biophys.* 2000;378:195–200.
34. Forbes RA, Steenbergen C, Murphy E. Diazoxide-induced cardioprotection requires signaling through a redox-sensitive mechanism. *Circ Res.* 2001;88:802–9.
35. Pain T, Yang XM , Critz SD, et al. Opening of mitochondrial K(ATP) channels triggers the preconditioned state by generating free radicals. *Circ Res.* 2000;87:460–6.
36. Tominaga M, Horie M, Sasayama S, et al. Glibenclamide, an ATP-sensitive K+ channel blocker, inhibits cardiac cAMP-activated Cl− conductance. *Circ Res.* 1995;77:417–23.
37. Wilde AA, Escande D, Schumacher CA, et al. Potassium accumulation in the globally ischemic mammalian heart: a role for the ATP-sensitive potassium channel. *Circ Res.* 1990;67:835–43.
38. Venkatesh N, Lamp ST, Weiss JN. Sulfonylureas, ATP-sensitive K+ channels, and cellular K+ loss during hypoxia, ischemia, and metabolic inhibition in mammalian ventricle. *Circ Res.* 1991;69:623–37.
39. Picard S, Rouet R, Duval D, et al. KATP channel modulators and myocardial damages induced by ischemia-reperfusion: membrane lipids injury and arrhythmias. *J Mol Cell Cardiol.* 1998;30:2613–21.
40. Tosaki A, Hellegouarch A. Adenosine triphosphate-sensitive potassium channel blocking agent ameliorates, but the opening agent aggravates, ischemia/reperfusion-induced injury: heart function studies in nonfibrillating isolated hearts. *J Am Coll Cardiol.* 1994;23:487–96.
41. Szendrei L, Turoczi T, Kovacs P, et al. Mitochondrial gene expression and ventricular fibrillation in ischemic/reperfused nondiabetic and diabetic myocardium. *Biochem Pharmacol.* 2002;63:543–52.
42. Koretsune Y, Marban E. Cell calcium in the pathophysiology of ventricular fibrillation and in the pathogenesis of postarrhythmic contractile dysfunction. *Circulation.* 1989;80:369–79.
43. Koretsune Y, Marban E. Mechanism of ischemic contracture in ferret hearts: relative roles of [Ca2+]i elevation and ATP depletion. *Am J Physiol.* 1990;258:H9–16.

VI

Am J Physiol Heart Circ Physiol 284: H1592–H1600, 2003.
First published January 9, 2003; 10.1152/ajpheart.00902.2002.

B-type natriuretic peptide limits infarct size in rat isolated hearts via K_{ATP} channel opening

Savio P. D'Souza,[1] Derek M. Yellon,[1] Claus Martin,[2] Rainer Schulz,[2] Gerd Heusch,[2] Annamaria Onody,[3] Peter Ferdinandy,[3] and Gary F. Baxter [4]

[1]*The Hatter Institute, University College London Hospitals and Medical School, London WC1E 6BT, United Kingdom;* [2]*Institute for Pathophysiology, Universitatsklinikum Essen, Essen 45122, Germany;* [3]*Department of Biochemistry, University of Szeged, Szeged H-6720, Hungary; and* [4]*The Royal Veterinary College, London NW1 0TU, United Kingdom*

Submitted 17 October 2002; accepted in final form 6 January 2003

D'Souza, Savio P., Derek M. Yellon, Claus Martin, Rainer Schulz, Gerd Heusch, Annamaria Onody, Peter Ferdinandy, and Gary F. Baxter. B-type natriuretic peptide limits infarct size in rat isolated hearts via K_{ATP} channel opening. *Am J Physiol Heart Circ Physiol* 284: H1592–H1600, 2003. First published January 9, 2003; 10.1152/ajpheart.00902.2002.—B-type natriuretic peptide (BNP) has been reported to be released from the myocardium during ischemia. We hypothesized that BNP mediates cardioprotection during ischemia-reperfusion and examined whether exogenous BNP limits myocardial infarction and the potential role of ATP-sensitive potassium (K_{ATP}) channel opening. Langendorff-perfused rat hearts underwent 35 min of left coronary artery occlusion and 120 min of reperfusion. The control infarct-to-risk ratio was 44.8 ± 4.4% (means ± SE). BNP perfused 10 min before ischemia limited infarct size in a concentration-dependent manner, with maximal protection observed at 10^{-8} M (infarct-to-risk ratio: 20.1 ± 5.2%, $P < 0.01$ vs. control), associated with a 2.5-fold elevation of myocardial cGMP above the control value. To examine the role of K_{ATP} channel opening, glibenclamide (10^{-6} M), 5-hydroxydecanoate (5-HD; 10^{-4} M), or HMR-1098 (10^{-5} M) was coperfused with BNP (10^{-8} M). Protection afforded by BNP was abolished by glibenclamide or 5-HD but not by HMR-1098, suggesting the involvement of putative mitochondrial but not sarcolemmal K_{ATP} channel opening. We conclude that natriuretic peptide/cGMP/K_{ATP} channel signaling may constitute an important injury-limiting mechanism in myocardium.

cGMP; ischemia-reperfusion; infarct size; preconditioning

NATRIURETIC PEPTIDES ARE RELEASED from many tissues in response to physiological and pathological stimuli. A-type (atrial) natriuretic peptide (ANP) and B-type (brain or ventricular) natriuretic peptide (BNP) are the predominant natriuretic peptides in mammalian myocardium, stored within secretory granules as propeptides (2, 6, 24). Release of propeptides and the cleaved products in response to dilatation of the cardiac chambers in conditions such as heart failure has been well described. Under such conditions, the classical endocrine actions of ANP and BNP include vasodilatation of some peripheral vascular beds and natriuresis (38, 39). These actions are mediated by elevation of intracellular cGMP after peptide binding to natriuretic peptide receptor type A (NPR-A), a membrane-bound particulate guanylyl cyclase (2, 6, 15, 29).

BNP is the principal natriuretic peptide in ventricular myocardium. Experimental and clinical evidence suggests that brief episodes of ischemia or hypoxia, insufficient to cause alterations in end-diastolic pressure or irreversible tissue injury, can evoke a rapid release of BNP from cardiac tissue. Hypoxic perfusion of isolated hearts led to a rapid increase of BNP immunoreactivity in coronary effluent (35). In patients undergoing percutaneous transluminal coronary angioplasty, coronary sinus BNP concentration increased rapidly after balloon deflation (33), and circulating plasma concentrations of BNP were elevated 4.5-fold in patients after episodes of unstable angina (32). A functional role for the rapid release of BNP in response to brief periods of myocardial ischemia is not known. The recognition in recent years that several neurohormonal mediators are released from myocardium during brief periods of ischemia underpins the current mechanistic model of ischemic preconditioning (1, 8, 28). Autocrine and paracrine mediators acting on G protein-coupled receptors, notably adenosine, bradykinin, opioid peptides, and catecholamines, participate in the activation of a multiple-stage signal transduction pathway. This involves opening of ATP-sensitive potassium (K_{ATP}) channels as either a downstream or proximal event essential for conferring resistance to a subsequent episode of ischemia. NPR-A does not couple through G proteins but, through elevation of cGMP, NPR-A activation could modulate K_{ATP} channel activity (16). Thus the signal pathway activated by BNP might represent an alternative prosurvival mechanism. To test the hypothesis that BNP is cytoprotective during ischemia-reperfusion through the opening of K_{ATP} channels, we examined the ability of exogenous BNP to limit irreversible myocardial injury, defining the involvement of K_{ATP} channel opening using pharmacological blockers of K_{ATP} channels.

Address for reprint requests and other correspondence: G. F. Baxter, Dept. of Basic Sciences, The Royal Veterinary College, Univ. of London, Royal College St., London NW1 0TU, UK (E-mail: gfbaxter@rvc.ac.uk).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

MATERIALS AND METHODS

Male Sprague-Dawley rats (300–400 g) were used for these studies. Animals were treated in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986. Rat BNP_{1-32} (hereinafter referred to as BNP), glibenclamide, sodium 5-hydroxydecanoate (5-HD), 8-bromo-cGMP (8-Br-cGMP), and triphenyltetrazolium chloride were from Sigma (Poole, UK). HMR-1098 was a gift of Aventis Pharma. All other reagents were of analytic standard.

Isolated Heart Preparation

Rats were anesthetized with pentobarbital sodium (50 mg/kg ip). Heparin (1 IU/g) was administered concomitantly. Excized hearts were perfused retrogradely through the aorta at 11.3 kPa with Krebs-Henseleit buffer [containing (in mmol/l) 118 NaCl, 25 $NaHCO_3$, 11 glucose, 4.7 KCl, 1.2 $MgSO_4 \cdot 7H_2O$, 1.2 KH_2PO_4, and 1.8 $CaCl_2 \cdot 2H_2O$; aerated with carbogen, pH 7.3–7.5, at 37°C]. Coronary flow rate (CFR) was determined by timed collection of the coronary effluent. A saline-filled latex balloon connected to a pressure transducer was inserted into the left ventricle (LV), and baseline end-diastolic pressure was set at 5–10 mmHg. Heart rate, LV end-diastolic pressure, and developed pressures were recorded continuously.

Infarct Size Evaluation

A 4-0 silk suture was positioned around the left main coronary artery and threaded through a plastic snare to permit reversible occlusion of the coronary artery. Coronary occlusion was induced for 35 min by clamping the snare onto the heart. Reperfusion was achieved by releasing the snare. At the end of 120-min reperfusion, the coronary artery was reoccluded, and the risk zone was delineated with Evans' blue. Hearts were sectioned (2 mm) and incubated in 1% triphenyltetrazolium chloride in phosphate buffer (pH 7.4, 37°C) for 15 min to define white necrotic tissue when fixed in 10% formalin for 24 h. Images of the sections were drawn by an operator blinded to the experimental treatment. Risk zone areas and infarct-to-risk ratios were determined by computerized planimetry (Planimetry+ version 1.0 for Windows).

Infarction Protocols

The experimental protocols for the three separate infarction studies are illustrated in Fig. 1.

Study 1: BNP concentration-response study. In the control group, hearts were stabilized for 15–20 min and then subjected to 35 min of regional ischemia, followed by 120 min of reperfusion. BNP (10^{-12}–10^{-8} mol/l) was added to the Krebs-Henseleit buffer, and perfusion was started 10 min before ischemia and continued until 30-min reperfusion.

Study 2: K_{ATP} channel blockade study. BNP (10^{-8} mol/l) was selected after the experiments described above. Hearts were randomized to one of the following experimental groups (Fig. 1): *1*) Control group, as described above. Six control hearts were perfused with 0.016% DMSO (the vehicle for glibenclamide). Because there was no effect on infarct size of DMSO, these hearts were combined for statistical evaluation with non-DMSO-treated control hearts to comprise *group 1*. *2*) BNP (10^{-8} mol/l) treatment, as described above. *3*) 5-HD (10^{-4} mol/l), a blocker of mitochondrial K_{ATP} channels (9, 36), was perfused 10 min before ischemia and continued until 30-min reperfusion. *4*) 5-HD (10^{-4} mol/l) + BNP (10^{-8} mol/l), coperfused as described above. *5*) Glibenclamide (10^{-6} mol/l), a nonselective blocker of sarcolemmal K_{ATP} and mitochondrial K_{ATP} channels (7, 17, 23, 37), was perfused 10 min before ischemia and continued until 30-min reperfusion. Glibenclamide was dissolved in DMSO (final concentration not more than 0.016%). *6*) Glibenclamide (10^{-6} mol/l) + BNP (10^{-8} mol/l), coperfused as described above. *7*) HMR-1098 (10^{-5} mol/l), a selective blocker of sarcolemmal K_{ATP} channels (14, 21), was perfused 10 min before ischemia and continued until 30-min reperfusion. *8*) HMR-1098 (10^{-5} mol/l) + BNP (10^{-8} mol/l), coperfused as described above.

Study 3: effects of 8-Br-cGMP. In the third infarct study, 8-Br-cGMP, a cell-permeable analog of cGMP, was perfused at 10^{-8}–10^{-5} mol/l, commencing 10 min before ischemia and continued until 30-min reperfusion. A control group was

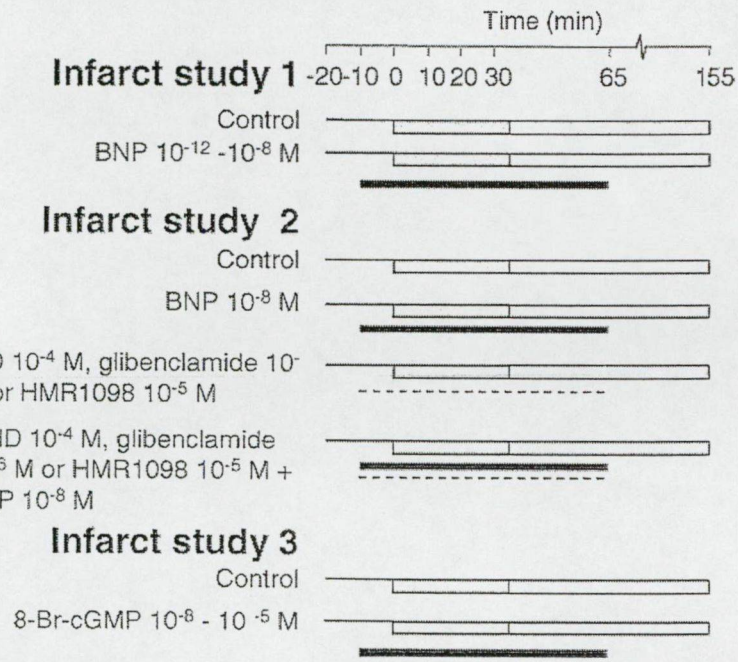


Fig. 1. Experimental protocols for infarct studies. Solid lines, time course of B-type natriuretic peptide (BNP) perfusion or 8-bromo-cGMP (8-Br-cGMP) perfusion; dotted lines, ATP-sensitive potassium (K_{ATP}) channel blocker perfusion; 5-HD, 5-hydroxydecanoate.

subjected to coronary artery occlusion and reperfusion only, as described above.

Myocardial cGMP Concentration

After stabilization, hearts were perfused for 10 min with BNP (10^{-12}–10^{-8} mol/l) as described above. Ventricular myocardium was then rapidly freeze-clamped to the temperature of liquid nitrogen. After extraction with trichloroacetic acid, the tissue cGMP concentration was determined by radioimmunoassay as described previously (5).

Endogenous BNP Release After Ischemia

After stabilization, during which baseline samples were collected, hearts were rendered globally ischemic for 2, 5, or 20 min. Flow was reinstituted, and the coronary effluent was sampled during reflow. Control hearts were perfused without ischemia. The ventricular tissue was immediately frozen in liquid N_2. Coronary effluent and ventricular tissue samples were analysed for BNP immunoreactivity by radioimmunassay using a commercially available kit [RIK 9085 BNP-32 (rat), Peninsula Laboratories; San Carlos, CA]. Tissue was extracted with trifluoroacetic acid. The peptide was purified on C18 columns, freeze-dried, and redissolved in "RIA buffer." RIA buffer concentrate was added to the samples of coronary effluent, and aliquots were used for the assay. On the basis of the competition of ^{125}I-labeled BNP and unlabeled BNP binding to a limited amount of specific antibodies, a standard curve was constructed from which the concentration of BNP in the samples was determined.

Statistical Analysis

Data are expressed as means ± SE. Infarct-to-risk ratios, risk zone volumes, and BNP tissue concentrations were analyzed using one-way ANOVA and Fisher's protected least-significant-difference post hoc test. LV function parameters, CFR, and BNP coronary effluent concentrations were evaluated using repeated-measures ANOVA with Bonferroni's post hoc test. Statistical significance between group means was defined as $P < 0.05$.

RESULTS

Technical Exclusions

A total of 217 animals was used. For the concentration-response infarct experiments, 65 hearts were used, of which 5 hearts were excluded: one was damaged by instrumentation, one had failure of the tetrazolium stain, and three had persistent bradyarrhythmia in the stabilization phase. In the second series of experiments, 55 hearts were used, of which 5 hearts were excluded: two failed to reperfuse, one had an instrumentation error that prevented precise LV function assessment, one had failure of the tetrazolium stain, and one had persistent bradyarrhythmia throughout reperfusion. In the third infarct series, 46 hearts were successfully perfused without exclusion. Therefore, we report the data for 156 successfully completed infarct experiments. An additional 36 hearts were used to examine tissue cGMP concentration, and 15 hearts were used to study the release of endogenous BNP.

Infarct Study 1: BNP Concentration-Response Study

The risk zone volumes were similar among all the groups (Table 1). The control infarct-to-risk zone ratio was 44.8 ± 4.4% without BNP treatment, consistent with previous results (20). Treatment with BNP limited infarct size in a concentration-dependent manner (Fig. 2). Significant limitation of infarction was observed with BNP (10^{-10}, 10^{-9}, and 10^{-8} mol/l). The highest BNP concentration studied (10^{-8} mol/l) resulted in the smallest infarct size (20.1 ± 5.2%, $P <$ 0.01 vs. control).

Preischemic global coronary flow rate averaged 12.5 ml/min among the six experimental groups. After coronary artery occlusion, there was a decrease in the global CFR of ~35% and a recovery to preischemic values immediately after reperfusion with gradual "rundown" during the remaining 120-min of perfusion (Fig. 3*A*). The global CFR measured at intervals throughout the protocol did not differ substantially among the experimental groups. There were no detectable differences among the groups in any of the parameters of LV function measured (spontaneous heart rate, developed pressure, or the rate-pressure product; data not presented). Developed pressure and rate-pressure product declined immediately after the onset of coronary occlusion to the same extent in all experimental groups.

Table 1. *Risk zone volume in the BNP concentration-response study (study 1), the K_{ATP} channel blocker study (study 2), and the 8-Br-cGMP study (study 3)*

Treatment Group	*n*	Risk Volume, mm^3
Study 1		
Control	11	353 ± 26
BNP (10^{-8} mol/l)	8	311 ± 26
BNP (10^{-9} mol/l)	9	309 ± 26
BNP (10^{-10} mol/l)	8	336 ± 33
BNP (10^{-11} mol/l)	12	323 ± 19
BNP (10^{-12} mol/l)	12	337 ± 15
Study 2		
Control	10	384 ± 32
BNP (10^{-8} mol/l)	5	345 ± 13
5-HD (10^{-4} mol/l)	6	339 ± 29
5-HD + BNP	6	312 ± 35
Glibenclamide (10^{-6} mol/l)	4	367 ± 36
Glibenclamide + BNP	6	370 ± 26
HMR-1098 (10^{-5} mol/l)	5	351 ± 40
HMR-1098 + BNP	8	348 ± 34
Study 3		
Control	13	320 ± 25
8-Br-cGMP (10^{-5} mol/l)	10	402 ± 14
8-Br-cGMP (10^{-6} mol/l)	9	315 ± 22
8-Br-cGMP (10^{-7} mol/l)	7	372 ± 34
8-Br-cGMP (10^{-8} mol/l)	7	329 ± 27

Values are means ± SE; *n*, number of experiments. BNP, B-type (brain or ventricular) natriuretic peptide; K_{ATP} channel, ATP-sensitive K^+ channel; 5-HD, 5-hydroxydecanoate; 8-Br-cGMP, 8-bromo-cGMP. No statistically significant differences between groups were detected (one-way ANOVA).

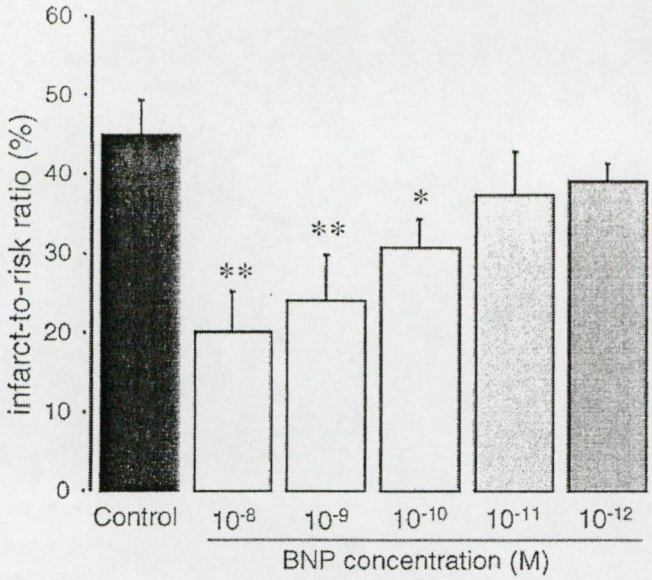


Fig. 2. Infarct-to-risk zone ratios for hearts perfused with BNP (10^{-12}–10^{-8} mol/l). Bars represent means ± SE; n = 8–12 experiments in each group. *P < 0.05 and **P < 0.01 vs. control (one-way ANOVA).

Infarct Study 2: K_{ATP} Channel Blockade Study

In the second series of infarct experiments, BNP was applied at a concentration of 10^{-8} mol/l and K_{ATP} channel blockers were coperfused with BNP.

The risk zone volume did not differ among the groups (Table 1). The control infarct-to-risk ratio was 47.1 ± 2.8% (Fig. 4). BNP (10^{-8} mol/l) treatment resulted in a significant limitation of infarct size (21.3 ± 2.8%, P < 0.01 vs. control). Coperfusion of BNP with either 5-HD or glibenclamide resulted in abolition of the protective effect of BNP (infarct-to-risk ratio: 52.3 ± 5.7% and 41.0 ± 7.1%, respectively, P = not significant vs. control). However, the infarct size limitation with BNP was not abolished by HMR-1098 (infarct-to-risk ratio: 14.7 ± 2.7%, P < 0.01 vs. control and P = not significant vs. BNP). None of the K_{ATP} channel blockers per se influenced infarct size.

As in *study 1*, the global CFR and LV function were not altered by BNP perfusion or any of the K_{ATP} channel blockers (Fig. 3*B*).

Infarct Study 3: 8-Br-cGMP Study

In the third series of experiments, 8-Br-cGMP was applied across a range of concentrations to examine the role of receptor-independent elevation of intracellular cGMP in myocardial responses to ischemia-reperfusion. Risk zone volume was similar among the experimental groups, and the control infarct-to-risk ratio was 38.7 ± 3.6%. We observed a paradoxical inverse concentration response with increasing concen-

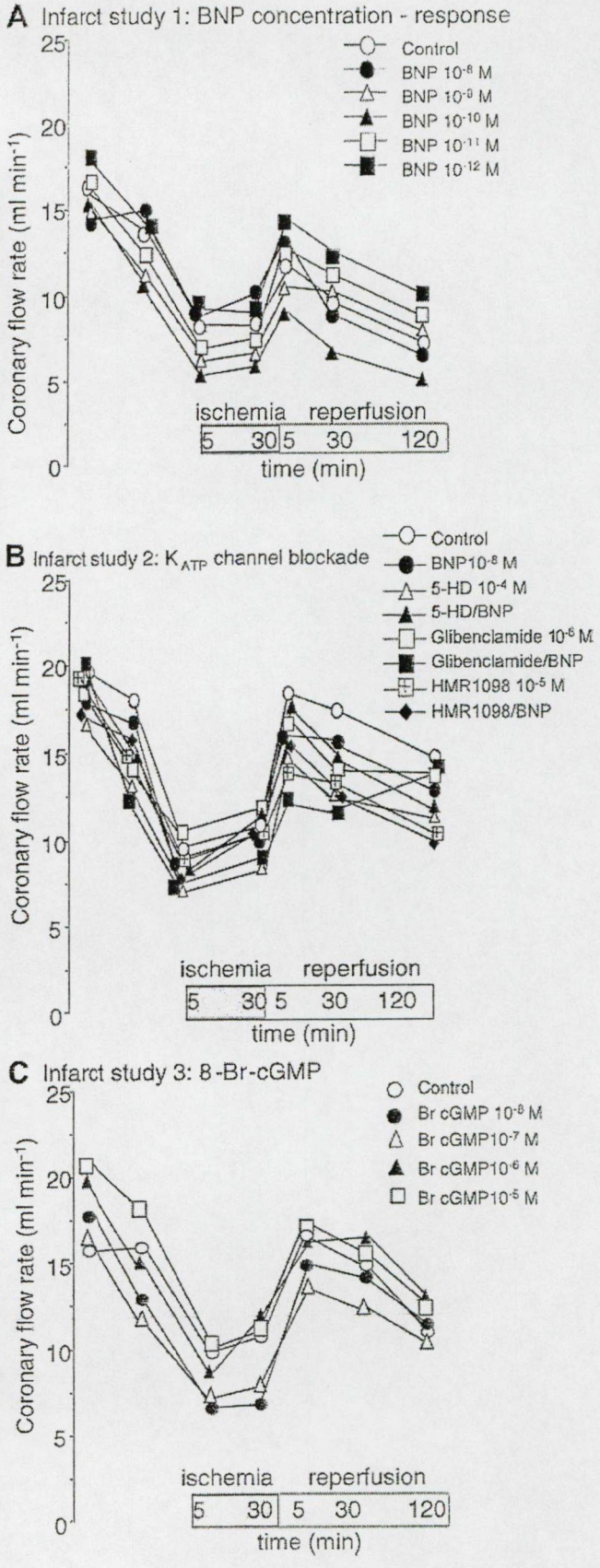


Fig. 3. Global coronary flow rate data for the BNP concentration-response study (*A*); K_{ATP} channel blockade study (*B*); and 8-Br-cGMP study (*C*). Symbols indicate mean values; SE bars have been removed for clarity.

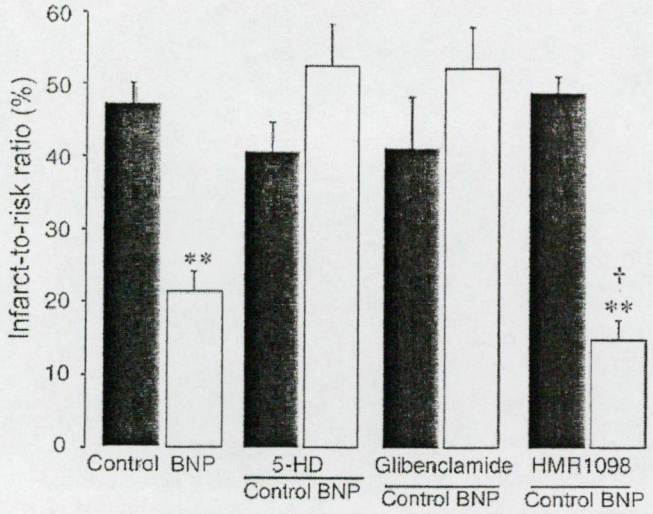


Fig. 4. Infarct-to-risk zone ratios for hearts perfused with K_{ATP} channel blockers with and without BNP (10^{-8} mol/l). Bars represent means ± SE; n = 4–10. **P < 0.01 vs. control; †P < 0.01 vs. HMR-1098 control (one-way ANOVA).

trations of 8-Br-cGMP (Fig. 5). Statistically significant limitation of infarction was observed with the lowest concentrations examined (10^{-7} mol/l: 23.1 ± 6.3%, P < 0.05 vs. control; 10^{-8} mol/l: 20.8 ± 4.2%, P < 0.05 vs. control). However, treatment with higher concentrations did not result in a significant limitation of infarction.

Perfusion with 8-Br-cGMP at 10^{-5} and 10^{-6} mol/l resulted in a nonsignificant increase in the preischemic CFR (Fig. 3*C*; 16.0 ± 1.0 ml/min in the control group vs. 19.5 ± 1.1 and 20.3 ± 1.1 ml/min in the 10^{-5} and 10^{-6} mol/l 8-Br-cGMP-treated groups, respectively, P = not significant). These influences of 8-Br-cGMP were not observed with the lower, infarct-limiting concentrations of the agent. No consistent patterns of 8-Br-cGMP treatment on LV contractility were observed during the perfusion protocol.

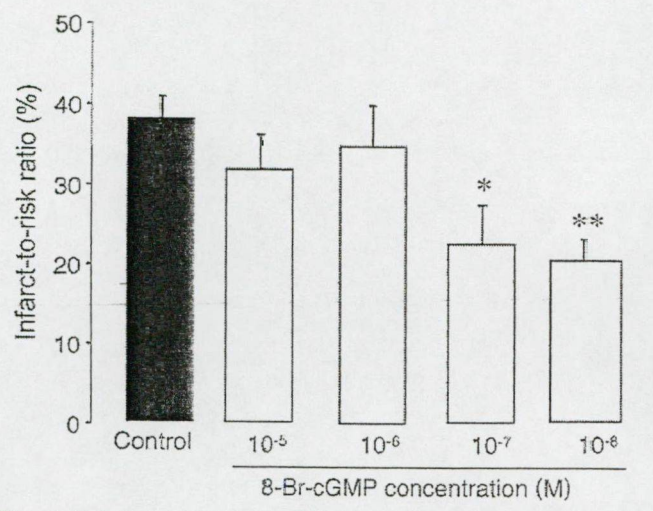


Fig. 5. Infarct-to-risk zone ratios for hearts perfused with 8-Br-cGMP. Bars represent means ± SE; n = 7–13. *P < 0.05 and **P < 0.01 vs. control (one-way ANOVA).

Effect of BNP on Myocardial cGMP Concentration

Perfusion with BNP for 10 min caused a concentration-dependent increase of the cGMP concentration in ventricular myocardium (Fig. 6). In control tissue, at the time point corresponding to the onset of myocardial ischemia, the cGMP concentration was 11.6 ± 0.5 pmol/g wet wt (n = 9). Significant increases in the tissue cGMP concentration were observed after perfusion with 10^{-9} mol/l BNP (cGMP: 20.3 ± 5.2 pmol/g wet wt, n = 5, P < 0.05) and 10^{-8} mol/l BNP (cGMP: 28.6 ± 1.5 pmol/g wet wt, n = 8, P < 0.01). Lower concentrations of BNP did not cause statistically significant elevation of the myocardial cGMP concentration.

BNP Release and Ventricular Myocardial BNP Concentrations

We observed a low basal release of BNP into the coronary effluent in the range of 1.1–9.7 pmol/l (see Fig. 7). In the control group (no ischemia), this efflux of BNP remained stable throughout 35 min of perfusion. Peak postischemic BNP concentrations in the coronary effluent were related to the duration of preceding ischemia: 2-min ischemia, 11.0 ± 2.4 pmol/l; 5-min ischemia, 20.1 ± 2.2 pmol/l; and 20-min ischemia, 41.5 ± 3.3 pmol/l (all P < 0.05 vs. control values; Fig. 7). Global ischemia for 2 and 5 min was not associated with changes in end-diastolic pressure above baseline. Hearts subjected to 20-min ischemia displayed an increase in end-diastolic pressure of ~10 mmHg above baseline values. Myocardial BNP concentration was 1.58 ± 0.16 pmol/g wet wt in control hearts and 1.77 ± 0.16, 3.69 ± 0.65 (P < 0.05 vs. control), and 1.82 ± 0.14 pmol/g wet wt, respectively, in hearts subjected to 2-, 5-, or 20-min global ischemia with 5-min reperfusion (n = 3–4 hearts/group).

DISCUSSION

The present investigation provides two principal new findings. First, acute infusion of exogenous BNP

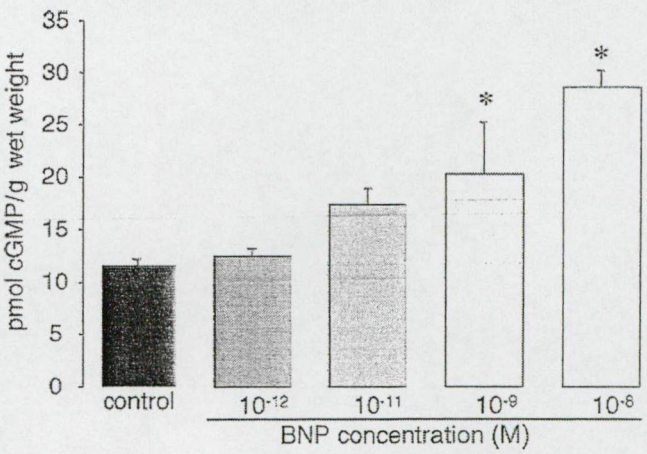


Fig. 6. Ventricular cGMP concentration after 10-min perfusion with BNP. Bars represent means + SE; n = 5–10. *P < 0.01 vs. control.

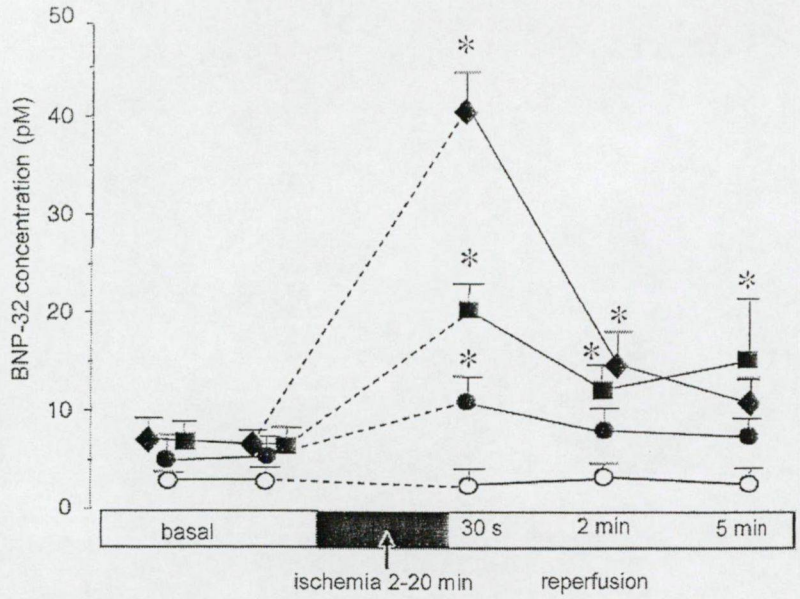


Fig. 7. Release of BNP into coronary effluent during control perfusion (○; $n = 3$), after 2-min ischemia (●; $n = 4$), 5-min ischemia (■; $n = 4$), or 20-min ischemia (◆; $n = 4$). * $P < 0.05$ vs. the corresponding control value (repeated-measures ANOVA).

is markedly protective against myocardial ischemia-reperfusion injury, leading to concentration-dependent infarct size limitation. Second, the mechanism of protection afforded by BNP is associated with elevation of cGMP and appears to involve K_{ATP} channel opening. The pharmacological selectivities of the widely used K_{ATP} channel blockers, applied at conventional inhibitory concentrations, may indicate involvement of the mitochondrial K_{ATP} channel rather than the sarcolemmal K_{ATP} channel. Although recent studies have reported that ANP (30) and the related noncardiac peptide urodilatin (27) limit infarct size in vivo, this is the first study to show the cardioprotective effect of BNP and to provide evidence of a primary mechanism of action of a natriuretic peptide on myocardium mediated by K_{ATP} channel activation.

The cell surface receptor mediating the biological actions of BNP is a particulate guanylyl cyclase A receptor, NPR-A, abundantly expressed in cardiac tissue (2, 29). Unlike receptors for adenosine, bradykinin, and opioids, the NPR-A receptor is not G protein coupled. This receptor contains an intracellular guanylyl cyclase catalytic domain that mediates most of the biological action of the natriuretic peptide through the conversion of GTP to cGMP (4, 13, 20). We observed significant increases in the ventricular cGMP concentration after 10-min perfusion with BNP (10^{-9} or 10^{-8} mol/l). Although this observation is consistent with NPR-A activation, we are unable to conclude at present that the infarct-limiting action of BNP in ischemic myocardium is a receptor-mediated action. Unfortunately, reliable NPR-A inhibitors such as HS-142-1 and A71915 are not available in sufficient quantity to undertake perfused heart or in vivo studies. Approaches using cultured cardiac myocytes may be feasible but may not accurately model endogenous protective mechanisms in intact tissue where there are interactions among several cell types and humoral mediators. Alternative approaches using mutant mouse strains with targeted deletion of either pro-BNP or NPR-A genes may also be possible in future studies. However, interpretation of findings from such animals, in the absence of complementary pharmacological data, may be clouded by the uncertainties of altered expression of other gene products, high levels of redundancy in signaling pathways, and the spontaneous development of cardiac pathology in these animals. For example, Izumi et al. (12) recently reported that mice with targeted deletion of the NPR-A gene sustained infarcts that were 20% smaller than wild-type control animals. However, the mutant animals had a substantial degree of LV hypertrophy. Moreover, interpretation of this study is predicated on the observation that natriuretic peptides upregulated vascular adhesion molecule expression in vitro, an effect that has not been demonstrated in humans or in intact animal models.

Our observation that the synthetic cGMP analog 8-Br-cGMP evoked, at low concentrations, an infarct-limiting effect similar to that observed with BNP is consistent with the notion that elevation of intracellular cGMP may indeed be a mechanism that is central to the cardioprotective action of BNP. cGMP elevation has been proposed to be a mechanism of injury limitation in ischemic myocardium (26), but the distal molecular mechanisms resulting in enhanced tolerance to ischemic injury associated with cGMP elevation are unclear. Proposed mechanisms include inhibition of L-type calcium channel opening (18), decreased intracellular concentrations of cAMP through a feedback mechanism and stimulation of cAMP phosphodiesterase (11, 18, 22), inhibition of the mitochondrial permeability transition pore (31), and opening of K_{ATP} channels (16). However, the unexpected finding that higher concentrations of 8-Br-cGMP (similar to those frequently applied in isolated cell and tissue pharmacol-

ogy) were not protective is intriguing and unexplained at present. Previous work has reported that high concentrations of cGMP are associated with cell injury. For example, Nakamura et al. (25) reported that the cytotoxic effects of a NO donor in a phaeocromocytoma line were augmented by a cell-permeable cGMP analog. A role of cGMP and cGMP-dependent protein kinase (cGK) in mediating apoptosis of pancreatic β-cells has been reported (19). Tepperman et al. (34) extended these observations to rat intestinal epithelial cells, showing that dibutyryl cCMP at millimolar concentrations reduced cell viability in culture. This cytotoxic effect of high intracellular concentrations of cGMP may be related to the generation of reactive oxygen species, because superoxide dismutase attenuated the injurious effects of the cGMP analog in intestinal epithelial cells. Thus our apparently paradoxical observation that increasing concentrations of 8-Br-cGMP were not associated with infarct limitation, whereas low concentrations were protective, may reflect an ambivalent effect of cGMP, both prosurvival and proinjury effects being mediated by this second messenger depending on concentration and pathophysiological context.

In myocardium, sarcolemmal K_{ATP} channels were originally postulated to participate in salvage from irreversible ischemia-reperfusion injury, because their opening would produce an increase in the outward potassium current leading to shortening of action potential duration, which would in turn reduce the Ca^{2+} influx through voltage-dependent Ca^{2+} channels and increase the time during which the Na^+/Ca^{2+} exchanger would operate to extrude Ca^{2+} from the cell. Since 1998, attention has focused on mitochondrial K_{ATP} channels in both ischemic preconditioning and pharmacological preconditioning studies (28). Much of the evidence implicating a role of mitochondrial K_{ATP} channels is reliant on the reputed selectivity of pharmacological agents such as 5-HD (a blocker of mitochondrial K_{ATP} channels) and HMR-1098 (a blocker of sarcolemmal K_{ATP} channels). With the caution that pharmacological specificity and selectivity may be subject to revision, our study provides pharmacological evidence for involvement of a K_{ATP} channel subtype, possibly a mitochondrial K_{ATP} channel, in the infarct-limiting action of BNP. Further studies in appropriate isolated cell and mitochondrial preparations using biophysical approaches are now being planned to probe the specific involvement of mitochondrial K_{ATP} channel opening.

While not constituting proof of mechanism, the association between concentration-dependent elevation of cGMP by BNP and infarct limitation that we observed leads us to hypothesize a role for cGK-I (protein kinase G). The cGMP/cGK-I pathway could promote K_{ATP} channel opening, representing an alternative signal cascade to the widely studied G protein receptor-coupled-protein kinase C pathway. Indeed, recent studies support the concept that K_{ATP} channel activation may be promoted by cGK in a variety of cell types, including ventricular myocytes (10). The contribution of cGK signaling to mitochondrial K_{ATP} channel activation and infarct limitation after BNP treatment will be the subject of further studies, using mice with targeted deletion of cardiac cGK-I.

BNP is a vasodilator in several vascular beds including coronary epicardial conductance arteries and coronary microvessels (3, 40) A surprising finding in our studies was the absence of any gross alterations in the global CFR secondary to BNP-mediated coronary vasodilatation. It likely that a more consistent and marked effect would be observed at higher concentrations than those used in our studies. The highest concentration of BNP we used (10^{-8} mol/l) is at the threshold for vasorelaxation in rat aortic rings (13). At present, we must conclude that the protective effect of BNP on ischemic myocardium is apparently independent of coronary vasodilatation or collateral vessel recruitment, because the rat heart is devoid of native coronary collateral vessels. It is of interest that in a previous study examining the coronary vasodilator mechanisms of ANP in a constant flow preparation, ANP reduced coronary perfusion pressure, an effect sensitive to inhibition by N^{ω}-nitro-L-arginine methyl ester (40). The possibility that BNP-associated cardioprotection may be related, at least in part, to NO generation and activation of soluble guanylyl cyclase is currently being investigated in our laboratory.

We observed that postischemic release of endogenous BNP increased in a graded fashion with ischemia severity. Moreover, the increase of tissue BNP after 2- and 5-min ischemia likely reflects cleavage of the stored propeptide in response to ischemia; after a 20-min ischemic stimulus, tissue levels of BNP were reduced as a consequence of massive release of the peptide. The immediate stimulus to BNP release could be either ischemia per se or local tissue deformation as a result of ischemia. At present, we are unable to comment on this except to say that we observed graded release of BNP after ischemia that was not associated with substantial changes in end-diastolic pressure. It is impossible to directly relate the concentrations of BNP in coronary effluent to the concentrations required to protect against infarction. Although the coronary effluent concentrations were two to three orders of magnitude less than the protective concentrations infused, local interstitial concentration during ischemia, when there is no flow and thus no washout, would be considerably higher than that detected in the coronary effluent during reflow. Thus changes in the coronary effluent concentrations reflect changes in the interstitial concentrations but cannot predict the interstitial concentrations.

In conclusion, this study is the first to demonstrate a cardioprotective effect of exogenous BNP against ischemia-reperfusion injury. The abrogation of this protective effect by glibenclamide and 5-HD, but not by HMR-1098, is consistent with, but does not constitute proof of, a mechanism involving opening of the putative mitochondrial K_{ATP} channel. Although we postulate that elevation of cGMP with activation of cGK-I is a plausible mechanism of K_{ATP} channel opening, the signaling pathway underlying this newly defined ac-

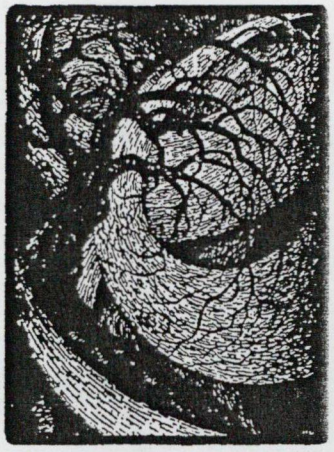
tion of BNP requires further elucidation, as does the involvement of NPR-A activation. Further studies in vivo and in other species are indicated to elucidate fully the cytoprotective potential of BNP and cGMP signaling in myocardial ischemia-reperfusion, especially the therapeutic application of recombinant BNP and inhibitors of neutral endopeptidase, the major enzymatic route for BNP degradation.

The authors gratefully acknowledge the support of British Heart Foundation Programme Grant RG/99002 and a cooperative award (to P. Ferdinandy and G. F. Baxter) from the Hungarian Ministry of Science and Technology and the British Council. S. P. D'Souza was supported by a grant from the Wellington Hospital Trust.

REFERENCES

1. **Baxter GF and Ebrahim Z.** Role of bradykinin in preconditioning and protection of the ischaemic myocardium. *Br J Pharmacol* 135: 843–854, 2002.
2. **Boomsma F and van den Meiracker AH.** Plasma A- and B-type natriuretic peptides: physiology, methodology and clinical use. *Cardiovasc Res* 51: 442–449, 2001.
3. **Brunner F and Wolkart G.** Endothelial NO/cGMP system contributes to natriuretic peptide-mediated coronary and peripheral vasodilation. *Microvasc Res* 61: 102–110, 2201.
4. **Chinkers M and Garbers DL.** Signal transduction by guanylyl cyclases. *Annu Rev Biochem* 60: 553–575, 1991.
5. **Csont T, Pali T, Szilvassy Z, and Ferdinandy P.** Lack of correlation between myocardial nitric oxide and cyclic guanosine monophosphate content in both nitrate-tolerant and -nontolerant rats. *Biochem Pharmacol* 56: 1139–1144, 1998.
6. **Espiner EA, Richards AM, Yandle TG, and Nicholls MG.** Natriuretic hormones. *Endocrinol Metab Clin North Am* 24: 481–509, 1995.
7. **Ferdinandy P, Szilvassy Z, Droy-Lefaix MT, Tarrade T, and Koltai M.** K_{ATP} channel modulation in working rat hearts with coronary occlusion: effects of cromakalim, cicletanine, and glibenclamide. *Cardiovasc Res* 30: 781–787, 1995.
8. **Gross GJ.** ATP-sensitive potassium channels and myocardial preconditioning. *Basic Res Cardiol* 90: 85–88, 1995.
9. **Grover GJ.** Protective effects of ATP sensitive potassium channel openers in models of myocardial ischemia. *Cardiovasc Res* 28: 778–782, 1994.
10. **Han J, Kim N, Joo H, Kim E, and Earm YE.** ATP-sensitive K^+ channel activation by nitric oxide and protein kinase G in rabbit ventricular myocytes. *Am J Physiol Heart Circ Physiol* 283: H1545–H1554, 2002.
11. **Hove-Madsen L, Mery PF, Jurevicius J, Skeberdis AV, and Fischmeister R.** Regulation of myocardial calcium channels by cyclic AMP metabolism. *Basic Res Cardiol* 91: 1–8, 1996.
12. **Izumi T, Saito Y, Kishimoto I, Harada M, Kuwahara K, Hamanaka I, Takahashi N, Kawakami R, Li Y, Takemura G, Fujiwara H, Garbers DL, Mochizuki S, and Nakao K.** Blockade of the natriuretic peptide receptor guanylyl cyclase-A inhibits NF-kappaB activation and alleviates myocardial ischemia/reperfusion injury. *J Clin Invest* 108: 203–213, 2001.
13. **Kambayashi Y, Nakao K, Kimura H, Kawabata T, Nakamura M, Inouye K, Yoshida N, and Imura H.** Biological characterization of human brain natriuretic peptide (BNP) and rat BNP: species-specific actions of BNP. *Biochem Biophys Res Commun* 173: 599–605, 1990.
14. **Kita H, Miura T, Miki T, Genda S, Tanno M, Fukuma T, and Shimamoto K.** Infarct size limitation by bradykinin receptor activation is mediated by the mitochondrial but not by the sarcolemmal K_{ATP} channel. *Cardiovasc Drugs Ther* 14: 497–502, 2000.
15. **Koller KJ and Goeddel DV.** Molecular biology of the natriuretic peptides and their receptors. *Circulation* 86: 1081–1088, 1992.
16. **Kubo M, Nakaya Y, Matsuoka S, Saito K, and Kuroda Y.** Atrial natriuretic factor and isosorbide dinitrate modulate the gating of ATP-sensitive K^+ channels in cultured vascular smooth muscle cells. *Circ Res* 74: 471–476, 1994.
17. **Leglenberg RJ, Houston RJ, Oeseburg B, and Smits P.** Effects of sulfonylurea derivatives on ischemia-induced loss of function in the isolated rat heart. *Eur J Pharmacol* 419: 85–92, 2001.
18. **Lohmann SM, Fischmeister R, and Walter U.** Signal transduction by cGMP in heart. *Basic Res Cardiol* 86: 503–514, 1991.
19. **Loweth AC, Williams GT, Scarpello JHB, and Morgan NG.** Evidence for the involvement of cGMP and protein kinase G in nitric oxide-induced apoptosis in the pancreatic B-cell line, HIT-T15. *FEBS Lett* 400: 285–288, 1997.
20. **Lucas KA, Pitari GM, Kazerounian S, Ruiz-Stewart I, Park J, Schulz S, Chepenik KP, and Waldman SA.** Guanylyl cyclases and signaling by cyclic GMP. *Pharmacol Rev* 52: 375–414, 2000.
21. **Manning Fox JE, Kanji HD, French RJ, and Light PE.** Cardioselectivity of the sulphonylurea HMR1098: studies on native and recombinant cardiac and pancreatic K_{ATP} channels. *Br J Pharmacol* 135: 480–488, 2000.
22. **Mery PF, Pavoine C, Belhassen L, Pecker F, and Fischmeister R.** Nitric oxide regulates cardiac Ca^{2+} current. Involvement of cGMP-inhibited and cGMP-stimulated phosphodiesterases through guanylyl cyclase activation. *J Biol Chem* 268: 26286–26295, 1993.
23. **Mocanu MM, Maddock HL, Baxter GF, Lawrence CL, Standen NB, and Yellon DM.** Glimepiride, a novel sulphonylureas, does not abolish myocardial protection afforded by either ischemic preconditioning or diazoxide. *Circulation* 103: 3111–3116, 2001.
24. **Nakamura S, Naruse M, Naruse K, Kawana M, Nishikawa T, Hosoda S, Tanaka I, Yoshimi T, Yoshihara I, and Inagami T.** Atrial natriuretic peptide and brain natriuretic peptide coexist in the secretory granules of human cardiac myocytes. *Am J Hypertens* 4: 909–912, 1991.
25. **Nakamura Y, Yasuda M, Fujimori H, and Kiyono M.** Cytotoxic effect of sodium nitroprusside on P.C12 cells. *Chemosphere* 34: 317–324, 1197.
26. **Opie LH.** Role of cyclic nucleotides in heart metabolism. *Cardiovasc Res* 16: 483–507, 1982.
27. **Padilla F, Garcia-Dorado D, Agullo L, Barrabes JA, Inserte J, Escalona N, Meyer M, Mirabet M, Pina P, and Soler-Soler J.** Intravenous administration of the natriuretic peptide urodilatin at low doses during coronary reperfusion limits infarct size in anesthetized pigs. *Cardiovasc Res* 51: 592–600, 2001.
28. **Schulz R, Cohen MV, Behrends M, Downey JM, and Heusch G.** Signal transduction of ischemic preconditioning. *Cardiovasc Res* 52: 181–198, 2001.
29. **Silberbach M and Roberts CT Jr.** Natriuretic peptide signalling: molecular and cellular pathways to growth regulation. *Cell Signal* 13: 221–231, 2001.
30. **Takagi G, Kiuchi K, Endo T, Yamamoto T, Sato N, Nejima J, and Takano T.** Alpha-human atrial natriuretic peptide, carperitide, reduces infarct size but not arrhythmias after coronary occlusion/reperfusion in dogs. *J Cardiovasc Pharmacol* 36: 22–30, 2000.
31. **Takuma K, Phuagphong P, Lee E, Mori K, Baba A, and Matsuda T.** Anti-apoptotic effect of cGMP in cultured astrocytes: inhibition by cGMP-dependent protein kinase of mitochondrial permeable transition pore. *J Biol Chem* 276: 48093–48099, 2001.
32. **Talwar S, Squire IB, Downie PF, Davies JE, and Ng LL.** Plasma N terminal pro-brain natriuretic peptide and cardiotrophin 1 are raised in unstable angina. *Heart* 84: 421–424, 2000.
33. **Tateishi J, Masutani M, Ohyanagi M, and Iwasaki T.** Transient increase in plasma brain (B-type) natriuretic peptide after percutaneous transluminal coronary angioplasty. *Clin Cardiol* 23: 776–780, 2000.
34. **Tepperman BL, Abrahamson TD, and Soper BD.** The role of cyclic guanylate monophosphate in nitric oxide-induced injury to rat small intestinal epithelial cells. *J Pharmacol Exp Ther* 284: 929–933, 1998.

35. **Toth M, Vuorinen KH, Vuolteenaho O, Hassinen IE, Uusimaa PA, Leppaluoto J, and Ruskoaho H.** Hypoxia stimulates release of ANP and BNP from perfused rat ventricular myocardium. *Am J Physiol Heart Circ Physiol* 266: H1572–H1580, 1994.
36. **Wang L, Cherednichenko G, Hernandez L, Halow J, Camacho SA, Figueredo V, and Schaefer S.** Preconditioning limits mitochondrial Ca^{2+} during ischemia in rat hearts: role of K_{ATP} channels. *Am J Physiol Heart Circ Physiol* 280: H2321–H2328, 2001.
37. **Workman AJ, MacKenzie I, and Northover BJ.** Do K_{ATP} channels open as a prominent and early feature during ischemia in the Langendorff-perfused rat heart? *Basic Res Cardiol* 95: 250–260, 2000.
38. **Yamamoto K, Burnett JC Jr, and Redfield MM.** Effect of endogenous natriuretic peptide system on ventricular and coronary function in failing heart. *Am J Physiol Heart Circ Physiol* 273: H2406–H2414, 1997.
39. **Yoshimura M, Yasue H, Morita E, Sakaino N, Jougasaki M, Kurose M, Mukoyama M, Saito Y, Nakao K, and Imura H.** Hemodynamic, renal, and hormonal responses to brain natriuretic peptide infusion in patients with congestive heart failure. *Circulation* 84: 1581–1588, 1991.
40. **Zellner C, Protter AA, Ko E, Pothireddy MR, DeMarco T, Hutchison SJ, Chou TM, Chatterjee K, and Sudhir K.** Coronary vasodilator effects of BNP: mechanisms of action in coronary conductance and resistance arteries. *Am J Physiol Heart Circ Physiol* 276: H1049–H1057, 1999.



VII

Am J Physiol Heart Circ Physiol 283: H69–H76, 2002.
First published February 28, 2002; 10.1152/ajpheart.00817.2001.

Nitrate tolerance does not increase production of peroxynitrite in the heart

TAMÁS CSONT,[1] CSABA CSONKA,[1] ANNAMÁRIA ÓNODY,[1] ANIKÓ GÖRBE,[1] LÁSZLÓ DUX,[1] RICHARD SCHULZ,[2] GARY F. BAXTER,[3] AND PÉTER FERDINANDY[1]
[1]*Cardiovascular Research Group, Department of Biochemistry, University of Szeged, Szeged, H-6720, Hungary;* [2]*Cardiovascular Research Group, Departments of Pharmacology and Pediatrics, University of Alberta, Edmonton, Alberta, Canada T6G 2R7; and* [3]*The Royal Veterinary College, University of London, London NW1 0TU, United Kingdom*

Received 17 September 2001; accepted in final form 26 February 2002

Csont, Tamás, Csaba Csonka, Annamária Ónody, Anikó Görbe, László Dux, Richard Schulz, Gary F. Baxter, and Péter Ferdinandy. Nitrate tolerance does not increase production of peroxynitrite in the heart. *Am J Physiol Heart Circ Physiol* 283: H69–H76, 2002. First published February 28, 2002; 10.1152/ajpheart.00817.2001.—Clinical studies have suggested that long-term nitrate treatment does not improve and may even worsen cardiovascular mortality, and the possible role of nitrate tolerance has been suspected. Nitrate tolerance has been recently shown to increase vascular superoxide and peroxynitrite production leading to vascular dysfunction. Nevertheless, nitrates exert direct cardiac effects independent from their vascular actions. Therefore, we investigated whether in vivo nitroglycerin treatment leading to vascular nitrate tolerance increases cardiac formation of nitric oxide (NO), reactive oxygen species, and peroxynitrite, thereby leading to cardiac dysfunction. Nitrate tolerance increased bioavailability of NO in the heart without increasing formation of reactive oxygen species. Despite elevated myocardial NO, neither cardiac markers of peroxynitrite formation nor cardiac mechanical function were affected by nitroglycerin treatment. However, serum free nitrotyrosine, a marker for systemic peroxynitrite formation, was significantly elevated in nitroglycerin-treated animals. This is the first demonstration that, although the systemic effects of nitroglycerin may be deleterious due to enhancement of extracardiac peroxynitrite formation, nitroglycerin does not result in oxidative damage in the heart.

nitroglycerin; nitric oxide; reactive oxygen species; myocardium

ISCHEMIC HEART DISEASE is a leading cause of death in developed countries and the quality of life of patients suffering from ischemic heart disease is significantly impaired. Organic nitrates including nitroglycerin have been used successfully in the treatment and prevention of ischemic heart disease for >100 years. However, development of tolerance to the vascular effects of nitrates due to long-term administration limits their clinical application (27). Moreover, recent clinical studies (17, 25) suggest that long-term nitrate treatment does not improve and may even worsen mortality due to cardiovascular reasons. Although the background of nitrate-induced increase in cardiovascular mortality is still not known, the possible role of nitrate tolerance has been suspected (17, 25).

Experimental evidence on the cellular mechanism of some of the adverse effects of nitrates have been described only in the past few years. Development of experimental vascular nitrate tolerance due to nitroglycerin treatment has been shown to increase the formation of reactive oxygen species (10, 24) and peroxynitrite in the vasculature, which leads to vascular dysfunction (9, 21). In light of these observations, treatment of patients with organic nitrate compounds may lead to side effects possibly due to generation of reactive oxygen species in the vasculature.

Most of the studies describing the possible oxidative effect of nitrates have looked at their vascular effects. There have been no attempts made so far to evaluate the effect of nitrates on the generation of reactive oxygen species directly in the heart. However, this may be the key missing information relevant to the unwanted effects of nitrates.

The reaction of nitric oxide (NO) with superoxide radical leads to the formation of peroxynitrite. The rate of this reaction is determined by the amount of NO and superoxide and the activity of superoxide dismutase (SOD) in the cellular microenvironment. Endogenous formation of peroxynitrite and reactive oxygen species is known to exert detrimental effects on myocardial function (1, 13, 14, 32). These findings suggest that similar to that in the vascular system, treatment with organic nitrates, especially during the development of nitrate tolerance, may result in oxidative stress and contractile dysfunction in the heart.

On the other hand, many studies (2, 6, 7, 15, 16, 26, 34, 37) show that NO donor compounds including nitroglycerin exert direct cardiac protective effects. We (7) have shown that nitroglycerin exerts a direct myo-

Address for reprint requests and other correspondence: P. Ferdinandy, Cardiovascular Research Group, Dept. of Biochemistry, Univ. of Szeged, Faculty of Medicine, Dóm tér 9, Szeged, Hungary H-6720 (E-mail: peter@bioch.szote.u-szeged.hu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Measurement of cardiac NO synthase, xanthine oxidoreductase, and SOD activities. To estimate endogenous enzymatic NO production, Ca^{2+}-dependent and Ca^{2+}-independent NO synthase activities in ventricular homogenates were measured by the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline as previously described (13). Powdered frozen ventricular tissue was placed in four volumes of ice-cold homogenization buffer (composition given in Ref. 33) and homogenized with an Ultra-Turrex disperser using three strokes of 20-s duration each. The homogenate was centrifuged (1,000 *g* for 10 min) at 4°C and the supernatant was kept on ice for immediate assay of enzyme activities. Samples were incubated for 25 min at 37°C in the presence or absence of EGTA (1 mM) or EGTA plus N^G-monomethyl-L-arginine (1 mM) to determine the level of Ca^{2+}-dependent and Ca^{2+}-independent NO synthase activities, respectively. NO synthase activities were expressed in picomoles per minute per milligram of protein.

Activity of xanthine oxidoreductase (xanthine oxidase and xanthine dehydrogenase), the major source of superoxide in rat hearts (11), was determined from ventricular homogenates by a fluorometric kinetic assay based on the conversion of pterine to isoxanthopterine in the presence (total xanthine oxidoreductase activity) and absence (xanthine oxidase activity) of the electron acceptor methylene blue, as described by Beckman et al. (4). Ventricular homogenates were prepared as for the measurement of NO synthase activity.

Total activity of SOD was measured by a spectrophotometric assay using a kit (Randox Laboratories). Approximately 100 mg ventricular tissue was homogenized in 10 volumes of ice-cold phosphate buffer (0.01 M, pH 7.0). SOD activity in homogenates was determined by the inhibition of formazan dye formation due to superoxide generated by xanthine and xanthine oxidase.

Measurement of markers of peroxynitrite. We measured dityrosine by spectrofluorometry in the perfusate and free nitrotyrosine by enzyme-linked immunosorbent assay (ELISA) in the perfusate and in the serum as markers of peroxynitrite generation (41, 42). ELISA (Cayman Chemical; Ann Arbor, MI) was conducted as described (13). Briefly, serum or perfusate samples were deproteinized by the addition of 4 volumes of ice-cold ethanol. After centrifugation, the supernatants were evaporated in nitrogen flow, dissolved in phosphate buffer, and incubated overnight with anti-nitrotyrosine rabbit IgG and nitrotyrosine acetylcholinesterase tracer in precoated (mouse anti-rabbit IgG) microplates, followed by development with Ellman's reagent.

Proteins containing nitrotyrosine residues were used as intracellular markers of peroxynitrite formation in ventricular sections obtained from control, nitrate tolerant, and positive control hearts. Positive controls were obtained by infusion of authentic peroxynitrite (1 mmol/l final concentration) for 10 min into the perfused hearts. The ventricles were frozen in isopentane cooled in liquid nitrogen and 6-μm-thick fresh frozen sections were made by a cryostate. The sections were fixed in acetone (5 min) and, after being washed, they were blocked for 15 min in Tris-buffered saline containing 1% bovine serum albumin, 0.1% Na-azide, and 0.5% Tween 20. Slices were incubated with a primary anti-nitrotyrosine antibody (rabbit polyclonal IgG, Upstate Biotechnology; Lake Placid, NY) for 2 h at room temperature. Bovine anti-rabbit IgG was used as a secondary antibody followed by fluorescein isothiocyanate-conjugated streptavidine labeling (DAKO; Glostrup, Denmark) (19). The sections were examined with the use of confocal laser scanning microscopy (Leica DMRE; Heidelberg, Germany).

Statistical analysis. Data were expressed as means ± SE and analyzed with unpaired *t*-test. $P < 0.05$ was accepted as indicating a statistically significant difference compared with the control group.

RESULTS

Vascular nitrate tolerance and cardiac function. To verify the development of nitrate tolerance after in vivo repetitive nitrate treatment, isolated aortic rings obtained from control and nitroglycerin-treated (9 × 100 mg/kg) animals were precontracted with an EC_{50} concentration of norepinephrine. The nitroglycerin concentrations needed to produce half-maximal relaxation were 0.09 ± 0.01 μmol/l in rings obtained from control animals versus 1.79 ± 0.28 μmol/l in nitroglycerin-treated group ($P < 0.05$, $n = 7$ in both groups). This 20-fold decrease in sensitivity to nitroglycerin observed in the present study is consistent with previous observations (6, 17, 35).

Parameters of cardiac performance in isolated working rat hearts, such as heart rate, aortic flow, coronary flow, left ventricular developed pressure, left ventricular end-diastolic pressure, and $\pm dP/dt_{max}$ were not affected by in vivo repetitive nitroglycerin treatment compared with the control group (Table 1).

In nontolerant rats (1, 5, or 12 h after treatment with a single dose of nitroglycerin), neither cardiac function (data not shown) nor nitroglycerin sensitivity of aortic rings (12-h group: EC_{50} of nitroglycerin 0.1 ± 0.02 μmol/l, $n = 5$, nonsignificant vs. control) were significantly changed. Nitroglycerin sensitivity of aortic rings was not further tested in the 1-h and 5-h groups.

Cardiac NO content and NO synthase. Cardiac NO content was significantly elevated in the nitroglycerin-tolerant group when measured by ESR spectroscopy after ex vivo spin trapping of NO in isolated hearts (Fig. 1*A*).

To test whether elevated NO derives from exogenous nitroglycerin or endogenous enzymatic sources, we measured cardiac activities of NO synthases. Endogenous enzymatic sources of NO, Ca^{2+}-dependent and Ca^{2+}-independent activities were not changed in the ventricular tissue due to development of nitrate tolerance (Fig. 1*B*).

Table 1. *Basal myocardial functional parameters in isolated working rat hearts*

	Control	Nitrate
Heart rate, beats/min	278 ± 7	285 ± 9
Coronary flow, ml/min	23.6 ± 0.8	23.8 ± 1.2
Aortic flow, ml/min	47.8 ± 1.8	51.8 ± 2.5
LVDP, kPa	18.9 ± 0.6	19.7 ± 0.4
LVEDP, kPa	0.54 ± 0.07	0.59 ± 0.05
$+dP/dt_{max}$, kPa/s	882 ± 43	898 ± 32
$-dP/dt_{max}$, kPa/s	405 ± 30	453 ± 39

Values are means ± SE; $n = 7$ isolated hearts per group. LVDP, left ventricular (LV) developed pressure; LVEDP, LV end-diastolic pressure; $\pm dP/dt_{max}$, first derivatives of LV pressure. Preload (1.7 kPa) and afterload (9.8 kPa) were kept constant throughout the perfusion.

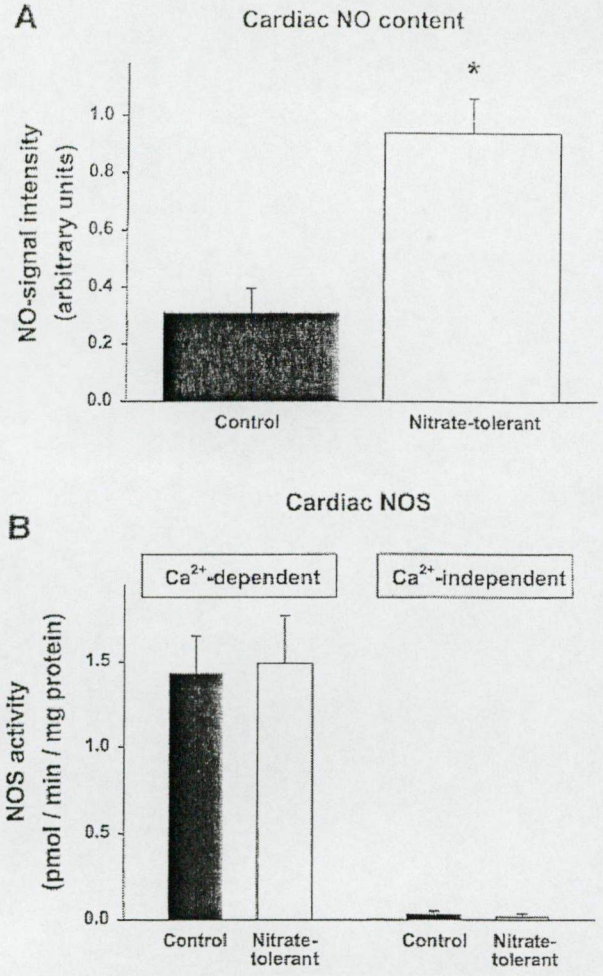


Fig. 1. Cardiac nitric oxide (NO) content (*A*) and Ca^{2+}-dependent and Ca^{2+}-independent NO synthase (NOS) activities (*B*) in ventricular tissue of control and nitroglycerin-treated rats. $^*P < 0.05$ vs. control (n = 4–7 in each groups).

Cardiac superoxide. To test whether nitrate tolerance increases cardiac superoxide generation, we performed lucigenin-enhanced chemiluminescence assay in fresh cardiac tissue. Cardiac superoxide generation remained unchanged due to repetitive nitroglycerin treatment compared with the control group (Fig. 2*A*). To further verify this negative finding, we measured the activity of xanthine oxidoreductase enzyme complex, a major enzymatic source of superoxide in rat hearts. There was no significant difference in activities of xanthine oxidase and xanthine dehydrogenase (Fig. 2*B*). We also tested the total activity of SOD in the heart, the major enzyme responsible for endogenous detoxification of superoxide. SOD activity was not changed in the nitroglycerin-tolerant hearts compared with controls (Fig. 2*C*).

Cardiac peroxynitrite. To estimate the peroxynitrite-generating capacity of the heart, hearts were isolated from nitroglycerin-tolerant and control rats and were perfused with a buffer supplemented with 0.3 mmol/l L-tyrosine. Formation of dityrosine and nitrotyrosine in the coronary effluent markers of peroxynitrite generation did not differ significantly between the two groups (Fig. 3*A*). To further study markers of peroxynitrite, we performed nitrotyrosine immunostaining in ventricular slices. Nitrotyrosine immunostaining detected by confocal laser scanning microscopy was negligible in hearts of both control and nitrate-tolerant groups, whereas an intensive nitrotyrosine staining was observed in positive control hearts, which were treated with authentic peroxynitrite (images not shown).

In hearts of nontolerant rats (1, 5, and 12 h after treatment with a single dose of nitroglycerin), formation of dityrosine in the coronary perfusate was not significantly different compared with controls (data not shown).

Cardiac hydroxyl radical. We also tested whether the formation of hydroxyl radical, a toxic derivative of peroxynitrite and superoxide, is changed in the heart as a result of development of nitroglycerin tolerance. Isolated hearts from control and nitroglycerin-tolerant

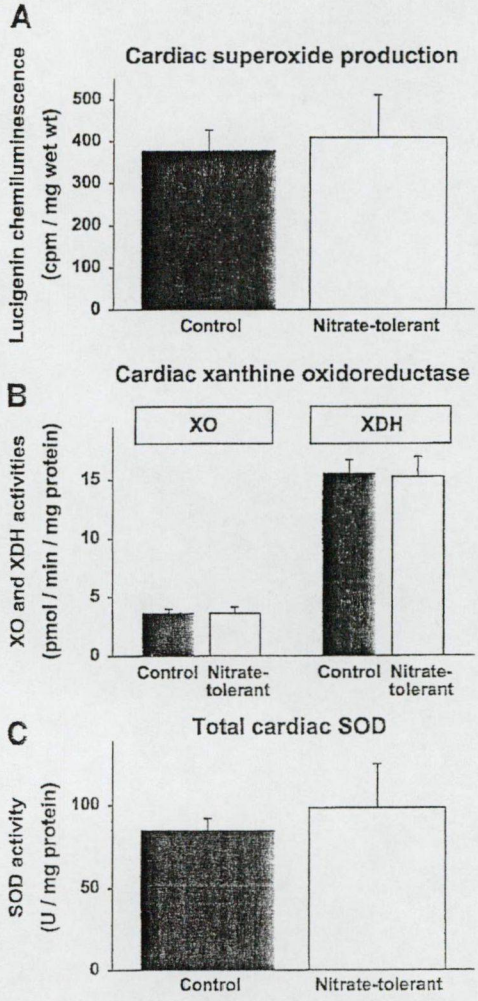


Fig. 2. Cardiac superoxide production (*A*), xanthine oxidase (XO), and xanthine dehydrogenase (XDH) activities (*B*), and superoxide dismutase (SOD) activity (*C*) in ventricular tissue of control and nitroglycerin-treated rats (n = 6–8 in each group).

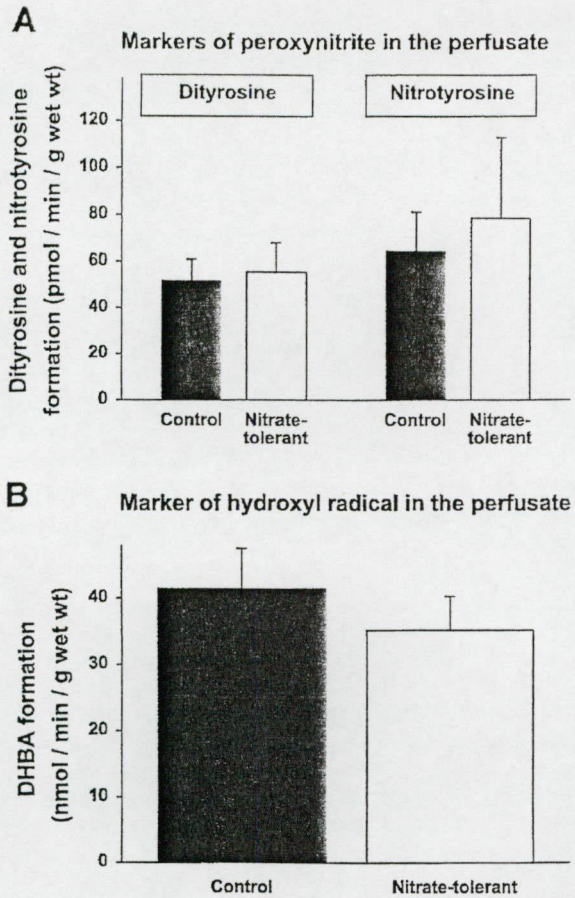


Fig. 3. Dityrosine and nitrotyrosine, markers of peroxynitrite (*A*), and 2,5-dihydroxybenzoic acid (DHBA), marker of hydroxyl radical (*B*), formation in perfusate from hearts perfused with 0.3 mmol/l L-tyrosine or 1 mmol/l salicylic acid in control and in vivo nitroglycerin-treated groups, respectively (n = 5–7 in each group).

rats were perfused in the presence of 1 mmol/l salicylic acid. Formation of 2,5-dihydroxybenzoic acid from salicylate in the perfusate due to hydroxyl radical activity was not significantly different (Fig. 3*B*).

Systemic peroxynitrite. Finally, we studied whether in vivo nitrate treatment increased formation of peroxynitrite anion in extracardiac tissues. Therefore, serum free nitrotyrosine concentration was measured in control and nitroglycerin tolerant animals, as well as in nontolerant rats treated with a single dose of nitroglycerin as a systemic marker for peroxynitrite. Serum free nitrotyrosine increased approximately twofold in nitroglycerin tolerant rats compared with controls (Fig. 4).

To test whether development of nitrate tolerance or nitrate treatment itself affects systemic peroxynitrite formation, serum nitrotyrosine was measured in nontolerant rats treated with a single dose of nitroglycerin. One hour after a single nitroglycerin treatment serum nitrotyrosine increased approximately twofold. However, there was no significant increase in serum nitrotyrosine level at 5 and 12 h after treatment (Fig. 4).

DISCUSSION

This is the first demonstration that repetitive nitrate treatment, which leads to the development of vascular nitrate tolerance, results in a sustained (>12 h) increase in serum nitrotyrosine. This, however, does not enhance production of reactive oxygen species including peroxynitrite in the heart. We have also shown here that nitroglycerin treatment increases the bioavailability of NO in the heart even when nitrate tolerance develops. We further observed that a single, acute treatment with nitroglycerin, which does not lead to nitrate tolerance, results in a transient (<5 h) increase in serum nitrotyrosine.

Many previous studies (9, 10, 22, 24) have suggested that nitrate treatment, especially when it results in the development of nitrate tolerance, is associated with increased free-radical generation in the vasculature. For instance, Münzel et al. (22) showed a twofold increase in total superoxide production and a significant decrease in vascular SOD activity in functionally damaged nitrate tolerant aortic rings. However, no studies have examined the influence of nitrate treatment and nitrate tolerance on cardiac generation of reactive oxygen species. In contrast to earlier studies on the vascular effects of nitrates and nitrate tolerance, here we have shown that the generation of cardiac superoxide, hydroxyl radical, and peroxynitrite, enzymatic superoxide synthesis by xanthine oxidoreductase and its detoxification by SOD, as well as the nonenzymatic breakdown of superoxide and peroxynitrite yielding hydroxyl radicals, were not changed in the heart after in vivo nitroglycerin treatment resulting in nitrate tolerance. Furthermore, myocardial mechanical function was not altered either as a result of vascular nitrate tolerance. This further supports the conclusion that superoxide and peroxynitrite genera-

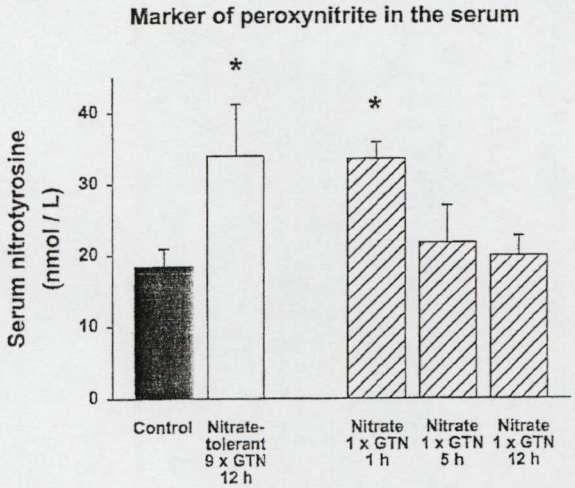


Fig. 4. Free nitrotyrosine concentration in deproteinized sera of control, nitroglycerin (GTN)-tolerant, and nontolerant nitroglycerin-treated rats. Serum samples were taken 12 h after repetitive nitroglycerin treatment in the tolerant group and 1, 5, or 12 h after single nitroglycerin treatment. *P < 0.05 vs. control, n = 5–7 in each groups.

tion in the myocardium and in the coronary vasculature were unaffected by nitroglycerin tolerance. Our data show that nitrate treatment does not enhance oxidative stress in the heart. It has been suggested that NO donors induce delayed preconditioning through the stimulation of free radical generation in the rabbit heart (26, 37). However, in those studies, a pharmacological approach (i.e., a single concentration of mercaptopropionyl glycine as a free radical scavenger) was used to investigate the involvement of free radicals in the cardioprotective effect of NO donors, and this may be less rigorous than biochemical measurements of several free radicals and their enzymatic production and breakdown as seen in the present study.

Although impairment of nitroglycerin bioconversion to NO and a decrease in NO bioavailability have been shown ex vivo in vessels from nitrate tolerant animals (23) and patients (31), these findings were refuted by others (20). Here we have demonstrated that cardiac NO accumulated after repetitive nitroglycerin treatment in vivo. This shows that nitrate treatment increases the bioavailability of NO in the heart even in the state of vascular nitrate tolerance. Because the cardiac activities of NO synthases did not change, the enhanced NO level must derive from nitroglycerin accumulated in the myocardium due to exogenous nitroglycerin treatment. This is in accordance with the observation by Torfgård et al. (39) showing the accumulation of nitroglycerin in cardiac tissue after long-term nitroglycerin treatment.

Increased cardiac NO due to nitrate treatment could result in enhanced formation of peroxynitrite in the present study. However, recent studies (18) suggest that peroxynitrite generation is mainly dependent on superoxide concentrations. Accordingly, the level of superoxide did not change after nitrate treatment in our experiments. Despite the increased level of cardiac NO, markers of peroxynitrite formation in the coronary perfusate (dityrosine and free nitrotyrosine) as well as in cardiac tissue (nitration of protein tyrosine residues) were not significantly affected by in vivo nitroglycerin treatment resulting in vascular nitrate tolerance. This shows that the generation of peroxynitrite either in the coronary vasculature or in the myocardium is not affected by nitrate treatment. Although enhanced vascular peroxynitrite formation has been reported in nitrate tolerance (9, 21), this was accompanied by increased superoxide generation in the vasculature. Nevertheless, in the heart (including coronary vasculature), we have not found any evidence of elevated superoxide or peroxynitrite formation. However, we did observe a significant increase in serum free nitrotyrosine concentration in nitrate-tolerant rats 12 h after the last nitroglycerin injection. This shows a sustained, increased production of peroxynitrite in extracardiac tissues, possibly in the extracardiac vasculature. This is in accordance with previous findings (9, 10, 21, 24) showing increased generation of reactive oxygen species in vascular tissue due to nitrate tolerance. To test whether increased generation of peroxynitrite is a general phenomenon of nitrate treatment or whether it is specific for nitrate tolerance, we also measured serum nitrotyrosine after a single dose of nitroglycerin, which does not lead to tolerance development. Increase in serum nitrotyrosine was found 1 h after administration of a single dose of nitroglycerin. However, this increase was not observed 5 and 12 h after treatment. This finding suggests that nitroglycerin treatment in the absence of nitrate tolerance leads to a transient increase in extracardiac peroxynitrite formation. This is in accordance with findings of Dikalov et al. (9), showing that the biotransformation of nitroglycerin is accompanied by superoxide formation in endothelial and smooth muscle cells in the absence of nitrate tolerance. The reason why nitrate tolerance extends the time frame of peroxynitrite formation is not clear; however, increased accumulation of tissue nitroglycerin is a plausible explanation (39).

Unfortunately, the endogenous formation of peroxynitrite cannot be directly detected in biological systems. Therefore, dityrosine and nitrotyrosine, products of the reaction between peroxynitrite and tyrosine, are the most often used markers of peroxynitrite, despite some criticism. Some in vitro biochemical data suggested that peroxynitrite does not cause tyrosine nitration at physiological pH (28), but this has been recently refuted (18, 30). Moreover, peroxynitrite-mediated nitration of tyrosine residues was proposed to be the most likely mechanism of nitrotyrosine formation in vivo (30). Myeloperoxidase activity in the presence of relatively high concentrations of nitrite may lead to nitrotyrosine formation (12). However, both myeloperoxidase activity and nitrite concentration were negligible because granulocyte-free, crystalloid-perfused hearts were used to test cardiac peroxynitrite formation in this study (13). In addition, concurrent measurement of nitrotyrosine and dityrosine decreases the possibility of false estimation of peroxynitrite generation (29). The possibility of enhanced myeloperoxidase activity in extracardiac tissues contributing to serum nitrotyrosine formation, however, cannot be excluded.

Clinical trials (17, 25, 38) have suggested that nitrate therapy may worsen the prognosis and survival in ischemic heart disease, although further studies are required to confirm these findings. Animal studies (9, 10, 22, 24) showed enhanced generation of oxygen free radicals and impaired vascular function associated with nitrate treatment. Moreover, vascular nitrate tolerance has been reported (36) to interfere with the cardioprotective action of classic preconditioning in rabbits in vivo. These observations may lead to a declining confidence in the use of nitrate therapy in patients. However, here we have provided several lines of evidence showing that in vivo nitroglycerin treatment leading to vascular nitrate tolerance does not result in oxidant stress and subsequent functional damage in cardiac tissue. Furthermore, we (7, 16) have shown previously that nitroglycerin exerts a direct anti-ischemic effect on the myocardium, which does not diminish even after the development of vascular nitrate tolerance. These findings may support the safety

of nitrate therapy even in the state of nitrate tolerance. However, nitroglycerin treatment, especially when leading to vascular nitrate tolerance, induces an increase in serum nitrotyrosine, a marker for systemic peroxynitrite formation. This shows that nitrate treatment may enhance oxidative stress in extracardiac tissues. The pathological significance of extracardiac oxidative stress due to nitrate treatment requires further investigation. Nevertheless, our results that nitroglycerin may induce oxidative stress in extracardiac tissue but not in the heart suggest that either cardioselective nitrates, nitrate compounds with additional antioxidant activity, or combination therapy with nitrates and antioxidants may increase the safety of organic nitrates in the chronic treatment of ischemic heart disease. Combined treatment with nitroglycerin and the antioxidant vitamin C has been used successfully to attenuate the development of nitrate tolerance in animal studies and humans (3, 8). Our present study opens a new perspective for "nitrate-antioxidant" treatment in ischemic heart disease.

In conclusion, in vivo nitroglycerin treatment, which results in vascular nitrate tolerance, may enhance oxidative stress in extracardiac tissues, but does not increase formation of oxygen free radicals and peroxynitrite in the heart. Moreover, it increases the bioavailability of cardiac NO. These findings support the safety of nitrate therapy even in the state of vascular nitrate tolerance although the importance and possible risk of nitrate-induced increase in extracardiac peroxynitrite generation requires further investigation. Our results provide a rationale for the development of cardioselective nitrates and promote further clinical studies on combination therapy with nitrates and antioxidants.

This work was supported by Hungarian Scientific Research Fund Grant T029843, Hungarian Ministries of Education and Health Grants FKFP-0340/2000, FKFP-0057/2001, and ETT-51/2000, Hungarian National R&D Program Grants NFKP-1/040 and NFKP-1/001, Hungarian North Atlantic Treaty Organization Cooperative Linkage Grant LST.CLG.976650, and by a cooperative grant from the British Council and the Hungarian Government. R. Schulz is a senior scholar of the Alberta Heritage Foundation for Medical Research.

REFERENCES

1. **Ambrosio G, Zweier JL, and Flaherty JT.** The relationship between oxygen radical generation and impairment of myocardial energy metabolism following post-ischemic reperfusion. *J Mol Cell Cardiol* 23: 1359–1374, 1991.
2. **Banerjee S, Tang XL, Qiu Y, Takano H, Manchikalapudi S, Dawn B, Shirk G, and Bolli R.** Nitroglycerin induces late preconditioning against myocardial stunning via a PKC-dependent pathway. *Am J Physiol Heart Circ Physiol* 277: H2458–H2494, 1999.
3. **Bassenge E, Fink N, Skatchkov M, and Fink B.** Dietary supplement with vitamin C prevents nitrate tolerance. *J Clin Invest* 102: 67–71, 1998.
4. **Beckman JS, Parks DA, Pearson JD, Marshall PA, and Freeman BA.** A sensitive fluorometric assay for measuring xanthine dehydrogenase and oxidase in tissues. *Free Radic Biol Med* 6: 607–615, 1989.
5. **Csonka C, Szilvássy Z, Fülöp F, Páli T, Blasig IE, Tosaki A, Schulz R, and Ferdinandy P.** Classic preconditioning decreases the harmful accumulation of nitric oxide during ischemia and reperfusion in rat hearts. *Circulation* 100: 2260–2266, 1999.
6. **Csont T, Páli T, Szilvássy Z, and Ferdinandy P.** Lack of correlation between myocardial nitric oxide and cyclic guanosine monophosphate content in both nitrate-tolerant and -nontolerant rats. *Biochem Pharmacol* 56: 1139–1144, 1998.
7. **Csont T, Szilvássy Z, Fülöp F, Nedeianu S, Páli T, Tosaki A, Dux L, and Ferdinandy P.** Direct myocardial anti-ischaemic effect of GTN in both nitrate-tolerant and nontolerant rats: a cyclic GMP-independent activation of K_{ATP}. *Br J Pharmacol* 128: 1427–1434, 1999.
8. **Dikalov S, Fink B, Skatchkov M, and Bassenge E.** Comparison of glyceryl trinitrate-induced with pentaerythrityl tetranitrate-induced in vivo formation of superoxide radicals: effect of vitamin C. *Free Radic Biol Med* 27: 170–176, 1999.
9. **Dikalov S, Fink B, Skatchkov M, Sommer O, and Bassenge E.** Formation of reactive oxygen species in various vascular cells during glyceryltrinitrate metabolism. *J Cardiovasc Pharmacol* 3: 51–62, 1998.
10. **Dikalov S, Fink B, Skatchkov M, Stalleicken D, and Bassenge E.** Formation of reactive oxygen species by pentaerithrityltetranitrate and glyceryl trinitrate in vitro and development of nitrate tolerance. *J Pharmacol Exp Ther* 286: 938–944, 1998.
11. **Downey JM, Hearse DJ, and Yellon DM.** The role of xanthine oxidase during myocardial ischemia in several species including man. *J Mol Cell Cardiol* 20, *Suppl* 2: 55–63, 1988.
12. **Eiserich JP, Hristova M, Cross CE, Jones AD, Freeman BA, Halliwell B, and van der Vliet A.** Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* 391: 393–397, 1998.
13. **Ferdinandy P, Danial H, Ambrus I, Rothery RA, and Schulz R.** Peroxynitrite is a major contributor to cytokine-induced myocardial contractile failure. *Circ Res* 87: 241–247, 2000.
14. **Ferdinandy P and Schulz R.** Peroxynitrite: toxic or protective in the heart? *Circ Res* 88: E12–E13, 2001.
15. **Ferdinandy P, Szilvássy Z, and Baxter GF.** Adaptation to myocardial stress in disease states: is preconditioning a healthy heart phenomenon? *Trends Pharmacol Sci* 19: 223–229, 1998.
16. **Ferdinandy P, Szilvássy Z, Csont T, Csonka C, Nagy E, Koltai M, and Dux L.** Nitroglycerin-induced direct protection of the ischaemic myocardium in isolated working hearts of rats with vascular tolerance to nitroglycerin. *Br J Pharmacol* 115: 1129–1131, 1995.
17. **GISSI-3.** Effects of lisinopril and transdermal glyceryl trinitrate singly and together on 6-week mortality and ventricular function after acute myocardial infarction. Gruppo Italiano per lo Studio della Sopravvivenza nell'infarto Miocardico. *Lancet* 343: 1115–1122, 1994.
18. **Goldstein S, Czapski G, Lind J, and Merenyi G.** Tyrosine nitration by simultaneous generation of (·)NO and O-(2) under physiological conditions. How the radicals do the job. *J Biol Chem* 275: 3031–3036, 2000.
19. **Krenács T and Rosendaal M.** Immunohistological detection of gap junctions in human lymphoid tissue: connexin43 in follicular dendritic and lymphoendothelial cells. *J Histochem Cytochem* 43: 1125–1137, 1995.
20. **Laursen JB, Mülsch A, Boesgaard S, Mordvintcev P, Trautner S, Gruhn N, Nielsen-Kudsk JE, Busse R, and Aldershvile J.** In vivo nitrate tolerance is not associated with reduced bioconversion of nitroglycerin to nitric oxide. *Circulation* 94: 2241–2247, 1996.
21. **Mihm MJ, Coyle CM, Jing L, and Bauer JA.** Vascular peroxynitrite formation during organic nitrate tolerance. *J Pharmacol Exp Ther* 291: 194–198, 1999.
22. **Münzel T, Hink U, Yigit B, Macharzina R, Harrison DG, and Mülsch A.** Role of superoxide dismutase in in vivo and in vitro nitrate tolerance. *Br J Pharmacol* 127: 1224–1230, 1999.
23. **Münzel T, Li H, Mollnau H, Hink U, Matheis E, Hartmann M, Oelze M, Skatchkov M, Warnholtz A, Duncker L, Meinertz T, and Forstermann U.** Effects of long-term nitroglycerin treatment on endothelial nitric oxide synthase (NOS III) gene expression, NOS III-mediated superoxide production, and vascular NO bioavailability. *Circ Res* 86: E7–E12, 2000.

24. **Münzel T, Sayegh H, Freeman BA, Tarpey MM, and Harrison DG.** Evidence for enhanced vascular superoxide anion production in nitrate tolerance. A novel mechanism underlying tolerance and cross-tolerance. *J Clin Invest* 95: 187–194, 1995.
25. **Nakamura Y, Moss AJ, Brown MW, Kinoshita M, and Kawai C.** Long-term nitrate use may be deleterious in ischemic heart disease: a study using the databases from two large-scale postinfarction studies. Multicenter Myocardial Ischemia Research Group. *Am Heart J* 138: 577–585, 1999.
26. **Nakano A, Liu GS, Heusch G, Downey JM, and Cohen MV.** Exogenous nitric oxide can trigger a preconditioned state through a free radical mechanism, but endogenous nitric oxide is not a trigger of classical ischemic preconditioning. *J Mol Cell Cardiol* 32: 1159–1167, 2000.
27. **Parker JD and Parker JO.** Nitrate therapy for stable angina pectoris. *N Engl J Med* 338: 520–531, 1998.
28. **Pfeiffer S and Mayer B.** Lack of tyrosine nitration by peroxynitrite generated at physiological pH. *J Biol Chem* 273: 27280–27285, 1998.
29. **Pfeiffer S, Schmidt K, and Mayer B.** Dityrosine formation outcompetes tyrosine nitration at low steady-state concentrations of peroxynitrite. Implications for tyrosine modification by nitric oxide/superoxide in vivo. *J Biol Chem* 275: 6346–6352, 2000.
30. **Reiter CD, Teng RJ, and Beckman JS.** Superoxide reacts with nitric oxide to nitrate tyrosine at physiological pH via peroxynitrite. *J Biol Chem* 275: 32460–32466, 2000.
31. **Sage PR, de La Lande IS, Stafford I, Bennett CL, Phillipov G, Stubberfield J, and Horowitz JD.** Nitroglycerin tolerance in human vessels: evidence for impaired nitroglycerin bioconversion. *Circulation* 102: 2810–2815, 2000.
32. **Schulz R, Dodge KL, Lopaschuk GD, and Clanachan AS.** Peroxynitrite impairs cardiac contractile function by decreasing cardiac efficiency. *Am J Physiol Heart Circ Physiol* 272: H1212–H1219, 1997.
33. **Schulz R, Nava E, and Moncada S.** Induction and potential biological relevance of a Ca2+-independent nitric oxide synthase in the myocardium. *Br J Pharmacol* 105: 575–580, 1992.
34. **Shah AM and MacCarthy PA.** Paracrine and autocrine effects of nitric oxide on myocardial function. *Pharmacol Ther* 86: 49–86, 2000.
35. **Silver PJ, Pagani ED, de Garavilla L, Van Aller GS, Volberg ML, Pratt PF, and Buchholz RA.** Reversal of nitroglycerin tolerance by the cGMP phosphodiesterase inhibitor zaprinast. *Eur J Pharmacol* 199: 141–142, 1991.
36. **Szilvássy Z, Ferdinandy P, Bor P, Jakab I, Szilvássy J, Nagy I, Lonovics J, and Koltai M.** Loss of preconditioning in rabbits with vascular tolerance to nitroglycerin. *Br J Pharmacol* 112: 999–1001, 1994.
37. **Takano H, Tang XL, Qiu Y, Guo Y, French BA, and Bolli R.** Nitric oxide donors induce late preconditioning against myocardial stunning and infarction in conscious rabbits via an antioxidant-sensitive mechanism. *Circ Res* 83: 73–84, 1998.
38. **Teo KK and Catellier DJ.** Long-term nitrate use in chronic coronary artery disease: need for a randomized controlled trial. *Am Heart J* 138: 400–402, 1999.
39. **Torfgård K, Ahlner J, Axelsson KL, Norlander B, and Bertler A.** Tissue levels of glyceryl trinitrate and cGMP after in vivo administration in rat, and the effect of tolerance development. *Can J Physiol Pharmacol* 69: 1257–1261, 1991.
40. **Tosaki A, Bagchi D, Páli T, Cordis GA, and Das DK.** Comparisons of ESR and HPLC methods for the detection of OH·radicals in ischemic/reperfused hearts: a relationship between the genesis of free radicals and reperfusion arrhythmias. *Biochem Pharmacol* 45: 961–969, 1993.
41. **Van der Vliet A, Eiserich JP, O'Neill CA, Halliwell B, and Cross CE.** Tyrosine modification by reactive nitrogen species: a closer look. *Arch Biochem Biophys* 319: 341–349, 1995.
42. **Yasmin W, Strynadka KD, and Schulz R.** Generation of peroxynitrite contributes to ischemia-reperfusion injury in isolated rat hearts. *Cardiovasc Res* 33: 422–432, 1997.